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Bernard, Hugo

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University of Dundee

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Ecole doctorate Biologie - Santé - Biotechnologies

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Hugo Bernard

ROLE OF p53 AND ITS ISOFORMS IN THE EXPRESSION OF FGF-2 AND TUMORAL NEOVASCULARIZATION

JURY

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The 30th of September, 2010

DECLARATION

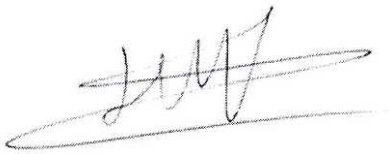
I declare that this thesis is based on results obtained from investigations which I, personally, have carried out and that the entire thesis is my own composition. Any work, other than my own, is clearly acknowledged with references to any relevant investigators or contributors. This thesis has not been previously presented, in whole or in part, for the award of any higher degree, and I have consulted all the references cited within the text.

Signed ……..

Date September 1st, 2010

Hugo Bernard

I confirm that Hugo Bernard has spent the equivalent of at least 3 years in research in my laboratory, Centre for Oncology and Molecular Medicine, Ninewells Hospital and Medical School, Dundee, UK and in the laboratory of Dr Anne-catharine Prats, Inserm Unite 858, Institut de Médicine Moléculaire de Rangueil, IFR31, 31432 Toulouse, France and that he has fulfilled the conditions of the University of Dundee and University Paul Sabatier Toulouse III thereby qualifying him to submit this thesis in application for the degree of Doctor of Philosophy.



Signed …… Signed

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Date September let, 2010 Date: September 1st 2010

Dr Jean-Christophe Bourdon Dr Anne-catharine Prats

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ROLE OF p53 AND ITS ISOFORMS IN THE EXPRESSION OF FGF-2 AND TUMORAL NEOVASCULARIZATION

Hugo Bernard

The tumour suppressor p53 actually exists as 9 protein isoforms. Among them, Δ133p53 α, β and γ result from the use of an alternative promoter and lack the N-terminal transactivation domain. In addition to its multiple functions maintaining cell integrity, p53 is also able to block angiogenesis, a process strongly contributing in tumour development. Here I have examined the role of p5 isoforms in the regulation of angiogenesis and tumor progression. I also focused my work on FGF-2 regulation by p53.

In a first part, full length p53 (p53) and/or Δ133p53 isoforms were selectively knocked-down with siRNAs in human glioblastoma cells U87. Conditioned medium produced by tumour cells knocked- down for Δ133p53 inhibited endothelial cell - EC - migration and tubulogenesis. Furthermore, in the chicken chorioallantoïd membrane CAM, Δ133p53 knockdown gave rise to smaller tumours devoid of vessels, whereas, in mice, it strongly inhibited tumour growth. Interestingly, the double knockdown of p53 and Δ133p53 also slowed town tumour growth in mice. Taqman Low Density Array revealed distinct gene expression profiles of pro and anti-angiogenic factors regulation following Δ133p53 and/or p53 knockdown. In particular, Δ133p53 knockdown resulted in specific down-regulation of Angiogenin and hepatocyte growth factor, whereas the main angiogenic factors FGF-2 and VEGF-A were not significantly affected.

Secondly we investigated the regulation of FGF-2 by p53 and its isoforms Δ133p53 in a human osteosarcoma cell line U2OS, at translational, transcriptional and secretion levels. It resulted in a sophisticated mode of regulation mediated by a transient IRES-dependent translation inhibition of FGF-2.

Our data reveal Δ133p53 isoforms as activators of angiogenesis and tumour progression, through a specific modulation of the angiogenic balance. These isoforms exhibit dominant-negative effect towards p53 but also intrinsic activities, while underlining the importance of considering Δ133p53 expression in cancers, as well as the potential antitumoural interest of drugs targeting this p53 isoform.

ROLE DE p53 ET DE SES ISOFORMES DANS LA REGULATION DE L'EXPRESSION DU FGF-2 ET DANS LA NEOVASCULARISATION TUMORALE

Hugo Bernard

Le suppresseur de tumeur p53 est exprimé sous forme de neuf isoformes. Parmi celles-ci, Δ133p53 α, β et γ sont issues de l'utilisation d'un promoteur alternatif induisant la perte du domaine de transactivation N-terminal. En supplément de ses multiples fonctions comme le maintien de l'intégrité cellulaire, p53 est également capable de bloquer l'angiogenèse, un processus contribuant fortement au développement tumoral. Au cours de ma thèse, j'ai analysé le rôle des isoformes de p53 dans la régulation de l'angiogenèse et dans la progression tumorale. J'ai également porté mes recherches sur la régulation de l'expression du FGF-2 par p53.

Tout d'abord, p53 pleine taille (p53) et/ou les isoformes Δ133p53 ont été disruptés spécifiquement par une approche de SiARN dans des cellules humaines de glioblastome - U87. En particulier, le milieu conditionné produit par les cultures traitées au SiΔ133p53 inhibe la migration des cellules endothéliales et leur tubulogenèse. De plus, dans un modèle de membrane chorioallantoïde de poulet, l'inhibition de l'expression de Δ133p53 provoque l'apparition de tumeurs U87 plus petites et dépourvues de vaisseaux. En parallèle, les mêmes cellules traitées implantées en sous cutané chez la souris croient beaucoup moins rapidement. De manière intéressante, le traitement double SiARN p53/Δ133p53 ralentit également le développement des tumeurs. Des experiences de Taqman Low Density Array ont montré des profils d'expression pro et anti-angiogéniques tres différents après traitement SiARN p53 et/ou Δ133p53. Le knock down de Δ133p53 révèle en particulier une inhibition de l'expression de l'Angiogénine et du HGF (facteur de croissance hépatique) alors que l'expression du FGF-2 et du VEGF-A ne sont pas modifiés de manière significative.

Dans une seconde partie j'ai étudié le rôle de la régulation du FGF-2 par p53 mais également par l'isoforme Δ133p53, dans un modèle de lignée cellulaire d'ostéosarcome humain U2OS, aux niveaux transcriptionnel, traductionnel et sécrétoire. J'ai mis en évidence un mode de régulation sophistiqué impliquant un inhibition transitoire de la traduction IRES-dépendente du FGF-2 suiteà l'inhibition de l'expression de p53.

Mes travaux de recherche ont permis la découverte d'un nouvel acteur de l'angiogenèse et de la progression tumorale, à savoir les isoformes Δ133p53, par un méchanisme spécifique de régulation de la balance angiogénique. Celles-cis démontrent un effet de dominant négatif envers p53 mais également des activités intrinsèques permettant ainsi de dévoiler leur importance dans les cancers et le potentiel thérapeutique de stratégies ciblant ces isoformes.

ABBREVIATIONS

ARN interférence	ARNi
Blood Vessels	Bvs
Checkpoint kinase	ChK
Chorio-Allantoic membrane	CAM
Chromatin immunoprecipitation	ChIP
Desoxyribonucleic acid	DNA
DNA binding domain	DBD
EncephaloMyocarditis virus	EMCV
Endoplasmic reticulum	ER
Endothelial Cells	ECs
Eukaryotic Initiation Factors	eiFs
Extra Cellular Matrix	ECM
Fibroblast Growth Factor 2	FGF-2
Glioblastoma Multiform	GBM
Guanosine Triphosphate	GTP
Heterogeneous Nuclear Ribonucleoprotein	hnRNP
High Molecular Weight	HMW
Hypoxia Inducible Factor-1α	HIF-1α
Immunohistochemistry	IHC
Internal Ribosome Entry Sites	IRES
IRES Trans-Acting Factor	ITAF
Low Molecular Weight	LMW
Messenger Ribonucleic Acid	mRNA
Mouse Double Minute 2	MDM2
Nuclear Localisation Signal	NLS
p53 core domain	p53C
p53 Responsive Element	p53RE
Polychain Reaction	PCR
Reactive Oxygen Species	ROS
Ribonucleic Acid	RNA
Simian Virus 40	SV40
Single DNA Strand Breaks	SSB
Smooth Muscle Cells	SMC

Transactivation Domain	TAD
Ultraviolet	UV
Untranslated region	UTR
Vascular Endothelial Growth Factor	VEGF

GLOSSARY

INTRODUCTION

A. p53

Prelude – The history of p53 protein characterization.

The most investigated protein in cancer – more than fifty thousands Pubmed results up to now – has been jointly discovered thirty years ago by four laboratories, all working on DNA viruses (reviewed in Kress *et al.* 1979; Lane and Crawford 1979 ; Linzer and Levine 1979; Melero *et al.* 1979; Finlay *et al.* 1988). In fact, Lane and Crawford were the first to publish and characterize the ability of the simian virus Large-T antigens to bind a host protein of a 53 kDa molecular weight, in hamster cells. These findings opened the widest field on oncology: the p53 world. But it all begun by a collegiate misunderstanding in the aspect that p53 was thought to be an oncogene.

The first studies revealed that not only the simian virus 40 – SV40 – large T antigen binds to p53 but other viral proteins from different viruses target it to transform cells (Rotter *et al.* 1980; Levine 1988). Although there were no evidence of the molecular key steps leading to cell transformation, experimental proof showed a link between DNA tumour viruses and use of proto-oncogene to form carcinomas (Lane and Benchimol 1990). Interestingly p53 was also found overexpressed in Non SV40 infected embryonic carcinomas (Linzer and Levine 1979). Several studies confirmed the relation between transformed cell/tumour and immunogenicity against p53 (DeLeo *et al.* 1979; Melero *et al.* 1979; Rotter *et al.* 1980; Crawford *et al.* 1982).

On another hand, two groups correlated p53 expression levels in lymphocytes and fibroblasts with DNA synthesis and overcome of G0/G1 and G1/S cell cycle transition stages (Milner and McCormick 1980; Mercer *et al.* 1984). p53 was upregulated by growth serum from a quiescent to a proliferate state, and particularly expressed in G1 and S phases (Reich and Levine 1984) Consistent with these results Shohat *et al.* in 1987 transfected p53 antisense plasmids in non-transformed NIH3T3 cells which showed a slower rate of DNA synthesis, as assayed by incorporation of [3H] thymidine (Shohat *et al.* 1987). All these findings supported the notion that wild type p53 was important for continuous cell proliferation. On the contrary, gross deletions of p53 gene in both alleles or even the 17q chromosome in which p53 gene is located, leading to irrevocably loss of p53 functions, indicated that p53 was not essential for cell growth (Wolf *et al.* 1984; Mowat *et al.* 1985).

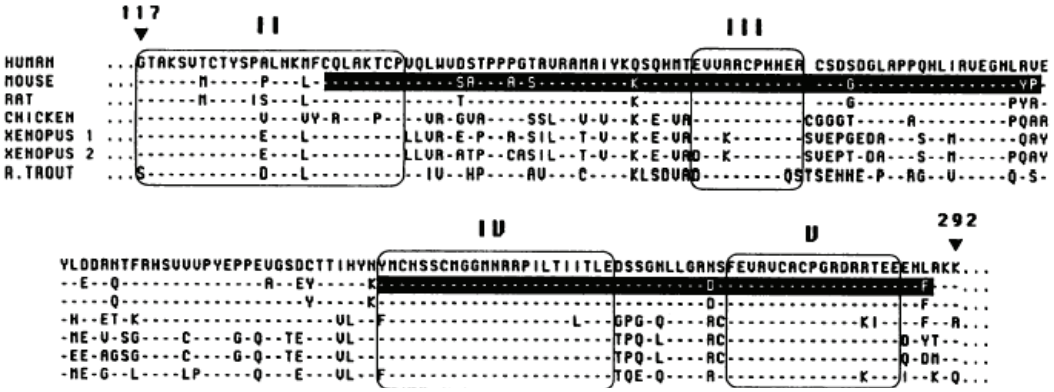
But the strongest evidence that really embedded this misbelief came from experiments using p53 plasmids transfection. Three different groups demonstrated that this p53 was one of the key steps leading to malignancy (Eliyahu *et al.* 1984; Jenkins *et al.* 1984; Parada *et al.* 1984; Jenkins *et al.* 1985). It could cooperate with an activated Ras to form foci from embryonic normal cells and tumours *in vivo.* Jenkins et al cloned the mouse p53 cDNA and immortalized finite lifespan cells. Importantly, Rotter's group showed by three different publications that non expressing p53 Ab-MuLV-transformed L12 cell line would only end up to be lethal in mice when transfected with p53 (Parada *et al.* 1984). These results confirmed that restoration of p53 in an overexpressed fashion would allow tumour formation.

As if research in virology was leading p53 field, the first discrepancies concerning oncogenicity of p53 rose from a study in Friend murine erythroleukemia. Theses cancers are induced by the integration of the spleen focus-forming virus – SFFV – provirus in few loci of the whole genome (Moreau-Gachelin *et al.* 1985). One of them frequently targeted the p53 gene, inactivating it (Mowat *et al.* 1985; Munroe *et al.* 1988). In all cases, the second allele was either lost through loss of the chromosome, or inactivated by deletion. In this tumour model, functional inactivation of the p53 gene seemed to confer a selective growth advantage to erythroid cells during the development of Friend leukemia *in vivo*.

A second set of studies highlighted some matter about the genetics of p53 gene. Two different approaches permitted to access the conserved sequence between species and showed p53 mutations in tumour cells. In fact, Levine's and Oren's groups used a mutated p53 cDNA clone, the clone 11-4, derived from F9 embryonic carcinoma cells to point out the fact that overexpression brought by stronger promoters from the original SV40 did not induce foci formation in Ras oncogene activated cells (Finlay *et al.* 1988). However, when inserting an inactivating sequence into the N-terminal of p53 protein, foci were formed. By recombination and analysis between the 11-4 clone and a mutant they found a single mutation – Cys to Phe – at position 132 essential for tumourogenicity. They also compared the 11-4 clone to the genomic sequence and found one mutation at position 135 – Val to Ala (Li *et al.* 2008). At this time they only considered it was a sequencing error or a polymorphism. The second team gathered multiple sequences from frog, mouse and human p53 gene (Soussi *et al.* 1987). They went to the conclusion that only five blocks in all three species had highly conserved amino-acid sequence. Moreover they showed that the frog p53 shared epitopes with

human p53. This mapping made up a useful tool in order to segregate point mutations activity. The same group published two years later a more complete mapping of these regions in several organisms – Figure 1 (Soussi *et al.* 1989). These findings permitted to settle wild type p53 core domains, succeeding to refund the p53 cDNA clone library.

Figure 1. Evolutionary conservation of p53 DNA-binding domain sequence.



From (Soussi *et al.* 1989). Part of the Core domain is shown here. Black areas show sites where the SV40 larget Antigen binds to p53. Open boxes represent the 4 of 5 cores DNA binding

It was now revealed that most studies used mutant p53 cDNA with transforming activity, but very few were known about wild type p53. Very shortly after these findings, two groups cleared this out, and announced its tumour suppressor activity (Baker *et al.* 1989; Finlay *et al.* 1989). Finlay *et al.* co-transfected wild type p53 with mutant p53 and activated Ras gene and observed a strong decrease in cell foci transformation while Baker *et al.* sequenced the p53 gene in tumours and found mutations in highly conserved regions. These experiments were the first to suggest that wild type p53 can negatively regulate cell growth and stop tumour formation.

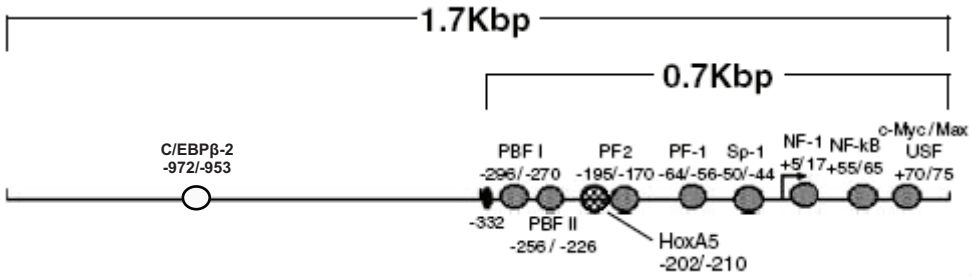
A.I. The p53 gene

The p53 gene is a 19.1 Kb length gene localized on the small arm of the chromosome 17 at locus 17p13.1 – NCBI ref: NC_000017.10. It contains eleven exons varying from 22 to 1268 bp and ten introns varying from 81 to 10000 bp (Lamb and Crawford 1986). In this study, they compared the human and mouse p53 gene sequences. Strikingly, they found a very conservative homology in the length and the structure of the exons.

A.I.1 The p53 promoter and its regulation

Initially the murine p53 promoter was thought to be quite short – 700 bp – but recent results extend it to a bit more than 1 Kb – Figure 2 (Boggs and Reisman 2006; Boggs and Reisman 2007). 75 % sequence identity is shared between mouse and human promoters, suggesting evolutionary conserved binding elements. Two human promoters were first described: one upstream the first exon and the second in the intron 1 (Tuck and Crawford 1989). This latter encodes for transcript of 1125 bp and is considered only as a pseudogene whose expression varies during terminal differentiation of myeloid leukemia cells. Research in our lab showed another promoter located in the intron 4, giving birth to N-truncated forms of p53, and a second transcription initiation site located fifty four nucleotides downstream the 5' exon site.

Figure 2. Map of murine p53 promoter



Numerous factors bind p53 promoter and modulate its activity: PBF1 and 2: prolamin box-binding factor-1 and 2, PF1 and 1: PHD zinc fingers protein 1 and 2, USF: upstream stimulatory factor, NF-1: neurofibromin 1, C/EBPβ-2: CCAAT/enhancer binding protein-β-2. From (Boggs and Reisman 2006)

A.I.2 p53 gene expression

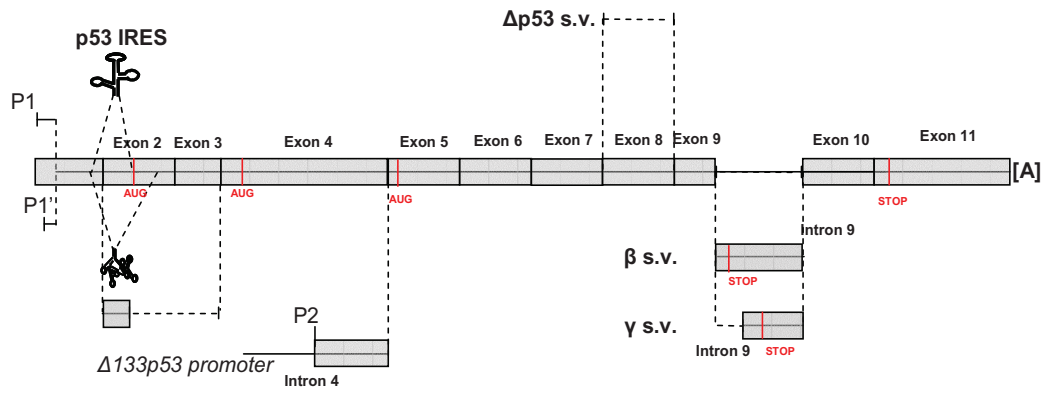
A.I.2.a. p53 transcripts.

If we summarize the results from different groups there are 30 different transcripts of p53 gene. In this part I will just summarize the findings upon the different p53 transcripts – Figure 3. A further analysis will be given later in this report. Two different groups identified in the 5' untranslated region characterized by Tuck and Crawford two translational regulating elements: Internal Ribosome Entry Sites – IRES – upstream the start codon – -140 to -1 – and also in part in the ORF – -140 to +39 (Ray *et al.* 2006; Yang *et al.* 2006). Three groups found an alternative translation initiation site in exon 4 following either alternative splicing or alternative translation initiation mechanism. Our laboratory identified an internal promoter in intron 4 which is consistent with the dual gene structure of the other p53 family members, p63 and p73. Additionally, they found two alternative splicings – β and γ – in intron 9 both containing stop codons. Finally Rohaly *et al.* detected an exon 8 truncated transcripts.

Nevertheless our lab has never confirmed the existence of a splicing event between exon 7 to 9 despite the use of specific primers (Bourdon *et al.* 2005; Rohaly *et al.* 2005).

Few investigations have been made on the 5'UTR of p53 gene but since the discovery of two IRESs it is believed that new effectors will soon be revealed. Human and murine p53 5' untranslated regions – 5'UTR – are predicted to form stable stem-

Figure 3. Structure of the human p53 gene



The figure is not scaled. Here are represented the three promoters P1, P1' and P2 in intron 4, The two IRESes, The first start codon and its alternative in exon 4 allowing expression of the Δ40p53 isoforms, Δ133p53 promoter followed by its 5'UTR and the start codon in exon 5, and EIIp53, Δp53, β and γ splice variants. For references refer to the p53 transcripts paragraph.

loop secondary structures inhibiting translation. p53 has been shown to *in vitro* bind its 5'UTR and inhibit its own translation by its RNA re-annealing properties and thus stabilizes the secondary structure (Mosner *et al.* 1995). More recently Takagi *et al.* identified two proteins regulating p53 mRNA translation: RPL26 enhanced it whereas nucleolin repressed it in response to DNA damage by Ionizing Radiations. These proteins are implicated in the folding and the stabilization of 5'UTR secondary structures (Takagi *et al.* 2005). The Polypyrimidine Tract-Binding protein – PTB – a well known IRES Trans-Acting Factor – ITAF – is also able to activate both p53 IRESs during doxorubicin treatment (Grover *et al.* 2008).

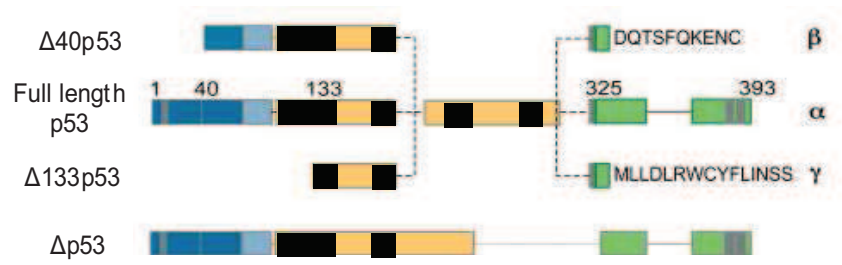
Little is known about the 3'UTR of the human p53 mRNA. p53 3'UTR is 1176 bp long and contains an Alu-like sequence of 470 nt that can inhibit p53 translation. To date, only few factors like Defective in Germ Line Development – GLD-1 – the C.elegans homolog of the mammalian protein Quaking and Hu antigen R – HuR – a RNA binding factor, have an effect on p53 translation (Mazan-Mamczarz *et al.* 2003; Schumacher *et al.* 2005). Cytoplasmic Polyadenylation Element-Binding protein – CPEB – is also able to promote p53 mRNA polyadenylation via its element in the

3'UTR inducing p53 and promoting thus p53-mediated senescence (Burns and Richter 2008). We can estimate that more proteins can bind to these elements, as Glyceraldehyde 3-Phosphate Dehydrogenase – GAPDH – and Heterogeneous Nuclear Ribonucleoprotein – hnRNP – D and A/B. Finally miR-125b is thought to affect DNA damage response by inhibition of p53 translation.

A.I.2.b. p53 translation regulation.

Fortunately, the 30 theoric mRNAs do not encode for 30 isoforms but 9 – 10 with the Δp53 isoform (Hollstein and Hainaut 2010). In this paragraph we only succinctly describe the p53 isoforms and the main domains involved – Figure 4. A more detailed overview will be done on the next paragraph. As this figure shows us, p53 is constituted by an N-terminal transactivation domain – TAD – with a proline rich domain, a DNA binding domain – DBD – where are represented in black squares four of the five highly conserved domains of p53 and a C-terminal domain composed of the Nuclear Localization Signal – NLS – the Nuclear Export Signal – NES – the oligomerization domain, and the extreme C-ter domain. The intron 9 spliced isoforms do not have an oligomerization domain, Δ40p53 isoforms lack part of the TAD and the Δ133p53 isoforms lack the whole TAD plus a small part of the DBD.

Figure 4. Structure of the human p53 gene



p53 encodes for 10 isoforms, namely p53α, p53β, p53γ, Δ40p53α, Δ40p53β, Δ40p53γ, Δ133p53α, Δ133p53β, Δ133p53γ and Δp53. From (Hollstein and Hainaut 2010)

A.II p53 structure and post translational modifications.

p53 is a 393 amino acid protein that acts as a tetrameric protein, mostly known in a homodimeric fashion but it is also able to form heterodimeric complexes with its isoforms. This is thought to modulate strongly its activity in response to different stresses. For a review refer to (Kruse and Gu 2009).

A.II.1 The N-Terminal region.

The N-terminal region of p53 consists first of two acidic unfolded Transactivation Domains – TAD1: residues 1 to 40; TAD2: 40-61 – and a proline rich

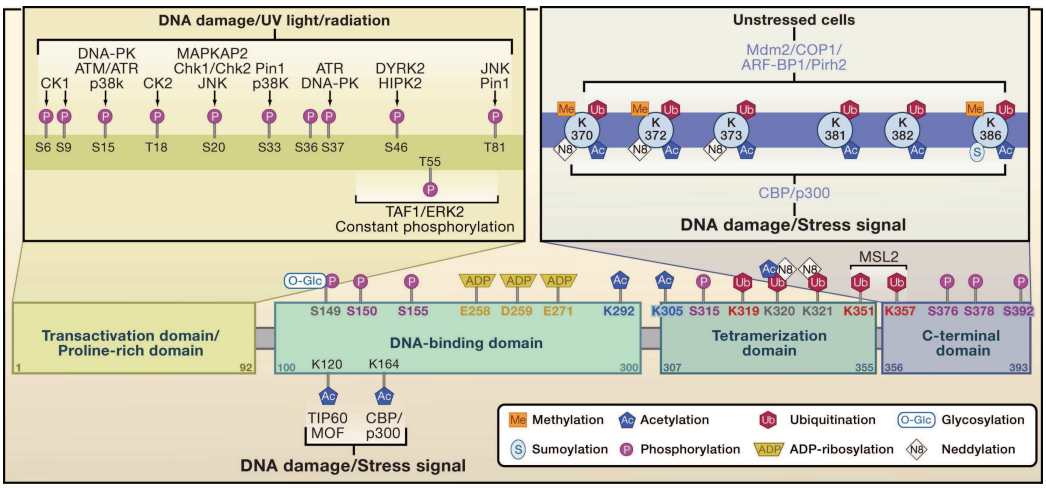
region. TADs are binding sites for numbers of interacting proteins from the transcription initiation complex, the transcription co-factors p300/CREB Binding Protein – p300/CBP – and Mouse Double Minute2 and 4 – MDM2/MDM4. Competition is also observed between co-factors like the overlap in MDM2 and p300 binding sites. p300/CBP is a histone acetyltransferase enzyme that loose chromatin structure to permit transcription initiation.

The p53 N-terminal domain is the place of multiple phosphorylation sites – S6-S9-S15-T18-S20-S33-S36-S37-S46-T55-T81 – Figure 5. These modifications enhance the binding affinity of several co-factors like Checkpoint kinase 1 and 2 – ChK1 and 2 – p38k, Ataxia-Telangiectasia Mutated protein – ATM – Ataxia-Telangiectasia and Rad3-related – ATR – DNA-dependent Protein Kinase – DNA-PK – Pin1 and others.

The proline rich domain is also characterized by numerous protein binding sites. It contains five PXXP motifs also known as Src Homology 3 – SH3 – domains. For example viruses like SV40 or adenoviruses use respectively their large-T and E1B-55 to inactivate p53. However the exact role of this region is not really understood and until now it is considered as a linker region between the TAD and the DBD.

A.II.2 The DNA Binding domain – DBD.

Figure 5. Post-translational modifications of the human p53 protein.



More than 10% of p53 residues are modified after specific inductions, and different modifications can occur in a single a.a. Most the modifications observed are present in the N-Ter and the C-Ter which are though to modulators of p53 activity. From (Kruse and Gu 2009).

The p53 core domain – p53C – consists of an immunoglobulin like β-sandwich with three series of loops and short helices that can be divided into two structural motifs that bind the minor and the major groove of a target DNA (Cho *et al.* 1994). Human

p53C is not very stable in comparison to its family proteins like p63. It is possible that this less stable structure is an evolution to tighter and more reactive folded-unfolded states in response to conditions changes, and also may permit plasticity to DNA binding events.

The p53C binds to a consensus sequence composed of two pairs of head to head pentamers forming half site decameric palindromes of 5'-RRRCWWGYYY-3' – R = A or G; W = A or T; Y = C or T – separated by between zero to thirteen base pair linker. This specific DNA sequence is also known as the p53 Responsive Element – p53RE – (el-Deiry *et al.* 1992; Bourdon *et al.* 1997). By ChIP paired-end diTag coupled sequencing to the whole genome, around 60% of known p53-responsive genes were identified with such consensus sequence without spacer (Wei *et al.* 2006). p53 protein binds DNA as a tetramer with higher affinity for genes involved in cell cycle arrest and DNA repair than those for apoptotic response (Weinberg *et al.* 2005).

Seven residues in the p53C are essential to bind the DNA half sites: K-120, S-241, R-248, R-273, A-276, C-277 and R-280 (Kitayner *et al.* 2006). Like the TAD, the DBD is susceptible to posttranslational modifications that enhance or inhibit the binding to DNA. Three Lysines in position 120, 164 and 292 can be acetylated. Three serines in position 149, 150 end 155 can be phosphorylated and amino-acid 256, 259 and 271 can be ADP ribosylated. A tight control of these modifications has been demonstrated as for example acetylation of residue 120 by Tat Interactive Protein 60 – TIP60 – after induction of an apoptotic stress (Tang *et al.* 2006).

A.II.3. The C-terminal domain

The C-terminal domain consists of a short β-strand and an α-helix. It contains the oligomerization domain in which is included a nuclear export sequence – a.a. 340 to 351 and 305 to 322 respectively – and a nuclear localization sequence, and an arginine and lysine rich basic region at the free C-terminal end shown to act as a nonspecific nucleotide binding region (Kim and Deppert 2006). The oligomerization domain allows p53 to form a homotetramer but also can do heterotetramers in combination with the Δ40p53 and Δ133p53 isoforms. The C-terminus domain of p53 binds non-specifically DNA by its Lysine residues with low affinity. In the same time, this region is involved in a sliding based mechanism for recognition of specific p53 target genes through the DNA molecule (McKinney *et al.* 2004).

The C-terminal domain is the target of multiple posttranslational modifications (Kruse and Gu 2009). Besides four phosphorylation sites in Serines 315, 376, 378 and 392, twelve Lysine residues in position 305, 319, 320, 321, 351, 357, 370, 372, 373, 381, 382 and 386 are particularly subject to modification like ubiquitination, acetylation, methylation, neddylation and sumoylation. These post-translational modifications allow uncovering the inhibiting effect of the C-terminus and allowing regulating specific binding to target p53 gene promoters (Sauer *et al.* 2008).

A.III. p53 Modes of action

Stress signals like DNA damage fire the sequential scheme of p53 activation that can be divided in three time dependent events: First p53 protein is rapidly stabilized upon post-translational modifications – phosphorylations, acetylation and inhibition of ubiquitination,… – allowing it to bind and induce expression of its target genes. Some of p53 target genes regulate p53 protein level – MDM2, Pirh2 – inducing thus a negative feedback loop (Levine *et al.* 2006). The post-translational modifications enables p53 to interact with a larger number of transcription co-factors and thus to transactivate a wider range of target genes. Finally when the cell damages are too important, multiple and complex modifications lead p53 to activate the programmed cell death.

A.III.1 Input signals for p53 activation

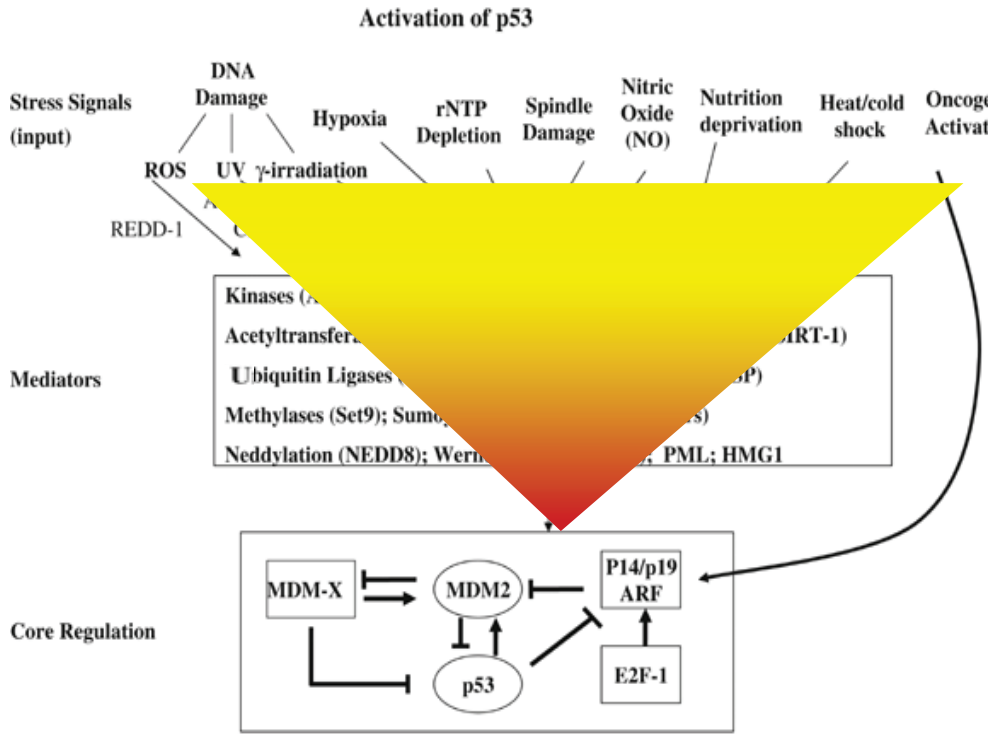
There is a large variety of stress signals that can induce p53 activation. It is certainly not the aim of this report to make an exhaustive list of all the factors and signals involved in p53 activation, but I considered rather relevant to synthesize and describe those which are relevant to my work. These stresses can be divided into groups involving different aspects of the cell homeostasis: DNA damage – or alteration – cellular starvation, aging, infections and inflammation, heat shocks, incorrect folding of proteins or activation of oncogenes (Levine *et al.* 2006) – Figure 6:

- Most of all, signals that trigger DNA alteration – gamma and UV irradiations, DNA cross linking, Reactive Oxygen Species – ROS – alterations of the desoxyribonucleotide or errors in replication – are the main signals of p53 activation. It has been thus called “the guardian of the genome” or the “gatekeeper cell growth and division” due to his strong ability to maintain the integrity of the genome and to control cell cycle progression (Lane 1992; Levine 1997). Each DNA damaging agents has its

own hallmark of p53 activation although two common pathways can be designed to explain the downstream activation of p53.

- Single DNA Strand Breaks – SSB – are due to replication stress, UV radiation and some chemicals and are recognized by a complex of three proteins Rad9, Rad1, Hus1 – not so randomly called 9-1-1 complex – which acts like a sliding clamp over DNA and by Rad17 with four Small Replication Factors – RFC – subunits – Rfc 2 to 5 – forming the RFC-related complex. At DNA damage site, Rad17-Rad2-5 regulates the recruitment of the 9-1-1 complex for the loading to the DNA. The 9-1-1-DNA complex catalyzes ATR phosphorylation activity and to a less extend the one of ATM (Niida and Nakanishi 2006). This mechanism is greatly enhanced by the binding at SSB of the Replication Protein A – RPA – which brings ATR and ATR Interacting Protein – ATRIP – to phosphorylate the 9-1-1 complex. ATR associates with other multiple mediators like Bloom syndrome protein – BLM – Histone H2A – H2AX – and p53 Binding Protein 1 – 53BP1 – complex to phosphorylate p53 but also MDM2 and chk1 kinase.

Figure 6. Upstream regulators and activators of p53 activity.



Input signal are hierarchically organized as a triangle: A consequent set of stimulus can activate five different families of mediators which in turn will modify the dynamics of the core domain in favour of p53 accumulation and activation. From (Levine *et al.* 2006).

- Double Strand Breaks – DSB – are DNA damage events caused by IR, changes in chromatin structure and chemicals. They can involve the MRN complex – MRE11-RAD1-NBS1 – and lead to the activation of ATM. ATM is able to phosphorylate itself and other mediators of the DSB response, amplifying its activation (Meek 2009). ATM is also potent for the indirect phosphorylation of DNA damage response effectors mediated by a complex of four proteins H2AX, Mediator of DNA Damage Checkpoint protein 1 – MDC1 – 53BP1, Structural Maintenance of Chromosomes protein 1 – SMC1 – or by direct phosphorylation of p53, MDM2 and ChK2 kinase (Sengupta and Harris 2005).

It is striking to observe that this whole system has been set up to exponentially amplify p53 activation by multiple pathways and means. This reinforces the essentiality of p53 in DNA damage response.

- Cell starvation is a wide sense term that enrolls multiples phenomena like nutrient or serum deprivation or hypoxia:

- Nutrient deprivation has been shown to activate AMP Activated Protein Kinase – AMPK – and phosphorylate p53 (Jones *et al.* 2005). Transient activation of AMPK pathway leads to G1/S arrest whereas persistent activation leads to senescence or apoptosis.

- The exact molecular link between low oxygen concentration and activation of p53 is still not clear, but strong evidence shows that the main actor is the Hypoxia Inducible Factor-1α – HIF-1α. In normoxic conditions, HIF-1α is rapidly hydroxylated by HIF Prolyl Hydroxylase Domain-containing enzymes – PHD – and leaded by the Von Hippel Lindau protein – pVHL – to E3 ubiquitin degradation. When O_2 levels does not permit hydroxylation of HIF-1α, this later will induce *via* hypoxia response elements transcription of a broad variety of genes involved in cell survival, proliferation and angiogenesis but also induces p53 stabilization (Koumenis *et al.* 2001; Fels and Koumenis 2005; Sanchez-Puig *et al.* 2005). Fels and Koumenis explain that in mild hypoxic conditions HIF-1α is stabilized but does not stabilize sufficiently p53 to induce a response. In more severe hypoxic conditions or anoxia – 0.2% and 0.02% of 02 – HIF-1α binds to stabilized p53, which in return starts a negative feedback loop inducing HIF-1α degradation mediated by MDM2 (Chen *et al.* 2003). Further studies in this direction showed that HIF-1α binds p53 through two domains, one in the oxygen-dependent degradation – ODD – domain (Hansson *et al.* 2002; Sanchez-Puig *et al.*

2005). These observations have raised controversies as other studies link p53 activation with hypoxia related stress such as acidosis, nutrient depletion or DNA damage (Kaluzova *et al.* 2004). For instance Pan *et al.* and Ravi *et al.* did not find p53 activation in hypoxic or anoxic conditions "alone" (Ravi *et al.* 2000; Pan *et al.* 2004). Regarding the formation of the HIF-1α-p53 complex, MDM2 plays an important role: MDM2 increases the expression of HIF-1α in normoxia and hypoxia, independently of p53 (Bardos *et al.* 2004; Zhang and Hill 2004). The MDM2 increase of expression in hypoxia remains unclear due to opposite results by different groups (Koumenis *et al.* 2001; Nieminen *et al.* 2005; Hubert *et al.* 2006). Other regulators of HIF-1α-p53 complex have been proposed: pVHL has been showed to induce a stabilization of p53 in hypoxia, thus avoiding the binding of MDM2 by several post translational modifications (Roe *et al.* 2006). Still, pVHL does not seem to be essential for p53 for the binding of HIF-1α (Schmid *et al.* 2004). Finally p300, known to interact with p53 and HIF-1α in a transcriptional competitive manner allows p53 transcription activity and therefore diminish the HIF-1α one (Blagosklonny *et al.* 1998; Schmid *et al.* 2004). The involvement of p300 may occur in anoxia because it supposes strong p53 levels.

This crosstalk allows us to apprehend better the mechanisms underlying the different responses to hypoxia. An important balance is p53 and HIF-1α expression levels, which bear some common targets regulated in an opposite fashion such as VEGF, GLUT-1 and Bcl-2. However this complex regulation is only partially conclusive. In the same time a specific pattern in p53 response encountered in hypoxia may be explained by Nitric Oxide – NO – levels, a severe-hypoxia-induced factor (Hammond and Giaccia 2006). For instance, it has been shown that hypoxia induces NO overproduction and overexpression by the Inducible Nitric Oxide Synthase – iNOS. This results in the induction of multiple pathways depending on NO concentrations (Thomas *et al.* 2008). From 100 nM to 300 nM NO induces HIF-1α, but in higher concentrations corresponding to very low oxygen concentration, NO is able to induce Serine 15 phosphorylation of p53 through the activation of ATM and ATR kinases. To note p53 creates a negative feedback loop with NO species: NO induces the stabilization of p53 that in consequence inhibit the NO synthase 2 or iNOS. This response avoids the stabilization of HIF-1α by NO (Forrester *et al.* 1996; Ambs *et al.* 1998). Indeed in strong hypoxic conditions, p53 is activated and binds HIF-1α and then directs it to the proteasome. Recently HIF-2α showed to regulate p53 levels in an

opposite fashion of HIF-1α (Bertout *et al.* 2009). As HIF2-α is also accumulated in normoxic conditions, in contrary to HIF1-α we can hypothesize that some competition between HIF 1 and 2 occurs for the binding of p53 protein. These elements could suggest a more complete and complex picture of p53 activation in hypoxic conditions.

- The immune system is also able to warn the cell from the rupture of homeostasis induced by infections or injury. It activates various immune agents like macrophages, mast cells, dendritic cells and promotes the release of cyokines, chemokines and ROS and Reactive Nitrogen Species – RNS. ROS and RNS – $OH^{\bullet}$, H_2O_2, $NO^{\bullet}$, $O_2^{\bullet}$, $OONO^-$ – are extremely active DNA damaging agents and thus induce p53 pathways. $NO^{\bullet}$ has a major role in inflammation. It is mostly produced by the iNOS and can have opposite effects depending on the levels of $NO^{\bullet}$ production. As postulated earlier $NO^{\bullet}$ can induce p53 phosphorylation and likewise MDM2 and HIF-1α is subject to a negative feedback loop with p53 in which p53 inhibit $NO^{\bullet}$ production by the blockade of iNOS.

A.III.2. Regulation of p53 protein

Although transcriptional regulation of p53 has been reviewed in this report, most of the p53 protein regulation occurs at the translational level. Indeed, p53 activation goes through first posttranslational modification of p53 and interaction with its co-factors promoting p53 stabilization, resulting in the uncovering of the DBD, then increase of its binding ability to specific target sequences by other posttranslational modifications and co-factors, and target gene activation by interacting with the general transcription machinery. In fact, it would be more relevant to consider also MDM2 to assess p53 regulation, because its interaction is a primordial element of p53 activity.

A.III.2.a. MDM2, the headman.

In a wide range of non stressed cells bearing a wild type p53 protein, its half life is included between 6 to 20 min, which is very short. This is due principally to its interaction with MDM2 and its subsequent poly-ubiquitination and degradation by the proteasome. MDM2, also called hdm2, is an E3 ubiquitin ligase of the Really Interesting New Gene – RING – finger family. The RING domain permits the direct binding of ubiquitin enzymes resulting in mono or poly-ubiquitination of p53 (Itahana K *et al.* 2007). Although MDM2 is the principal E3 ligase that induces p53 degradation – underlined by the early lethality of the MDM2-/- mice due to p53 dependent apoptosis – other E3 ligase like Caspase Recruitment Domain-Containing Protein 16 – CRDCP16

or COP1 – Pirh2 and Arf-BP1 for example have been recently described to have a p53 ubiquitination activity (Leng *et al.* 2003; Dornan *et al.* 2004; Chen *et al.* 2005). Therefore, it is not surprising to found several regulators of the p53-MDM2 interaction, such as p19 ^{ARF} or HAUSP (Zhang *et al.* 2003; Dai and Lu 2004; Li *et al.* 2004). Inversely cofactors can act negatively on p53 by promoting the MDM2-p53 interaction. In first line MDMX – also named MDM4 – stabilize both MDM2 and p53, increasing the ubiquitination and degradation of p53 (Meulmeester *et al.* 2005).

A.III.1.b. Other p53 regulators

Beside cofactors that directly target MDM2 activity and binding to p53, multiple cofactors are involved in direct or indirect stabilization of p53. An updated list of p53 posttranslational modifiers can be found at http://www.bnl.gov/biology/People/Anderson.asp.

As discussed above, four principal kinases phosphorylate p53: ATM, ATR, Chk1 and Chk2. However the number of these kinases can be far extended. Most of these kinases have the particularity to be able to phosphorylate p53 at multiple sites, rising up the complexity of p53 regulation. In order to regulate the activation of p53 by phosphorylation, the action of phosphatases has been described. For example, the Protein Phosphatase 2 – formerly PP2A – and Protein Phosphatase Magnesium Dependant 1 variant 430 – PPM1D430 – carry on the dephosphorylation of Ser46 and Ser15 of p53 (Chuman *et al.* 2009; Mi *et al.* 2009).

Histone acetylases are important regulators of p53 stability. Besides acetylation of p53 they also are involved in acetylation of the histones in the site of its target genes like CNP/300, Tip60 and hMof, thus synergically enhancing transcription (Gu *et al.* 1997). In addition, p300/CBP Associated Factor – PCAF – is another acetyltransferase of lysine 320 of p53 (Sakaguchi K *et al.* 1998).

p53 can be deacetylated by Histone Deacetylase – HDAC – complexes containing HDAC1 or Sir2α/Sirt1 (Luo *et al.* 2000; Luo *et al.* 2001). Deacetylation represses p53 dependent transcription activation, and thereby apoptosis and growth arrest. In vivo studies showed the importance of Silent Mating-Type Information Regulation-2 ortholog 1 – Sirt1 – in mice as Sirt1-/- mice presented hyperacetylated p53 and hypersensitivity to apoptosis following radiation. This study also suggests that acetylations can occur in non stressed cells and that acetylation/deacetylation mechanism is a dynamic event.

Deacetylation and dephosphorylation are quick-acting mechanisms and require little energy for the cells, on the contrary of the transcription mechanism. Therefore they are useful tools to stop p53 function when inputs signals are no longer present.

p53 protein is also modified by less known processes like methylation, neddylation and sumoylation. To date, three different methyltransferases have been described. Set7/9 monomethylates p53 at Lysine372 and actives it, Smyd2 and Set8/PR-Set7 act oppositely by monomethylation of lysines 370 and 382 (Chuikov *et al.* 2004; Huang *et al.* 2006; Shi *et al.* 2007). Once Lysines 370 and 382 are methylated, 53BP1, which is involved in DNA repair, strongly enhance its binding to p53. To note, a demethyltransferase has been described, the Amine Oxidase Flavin Containing Domain 2 – LSD1 – that demethylates one of these lysines (Huang *et al.* 2007).

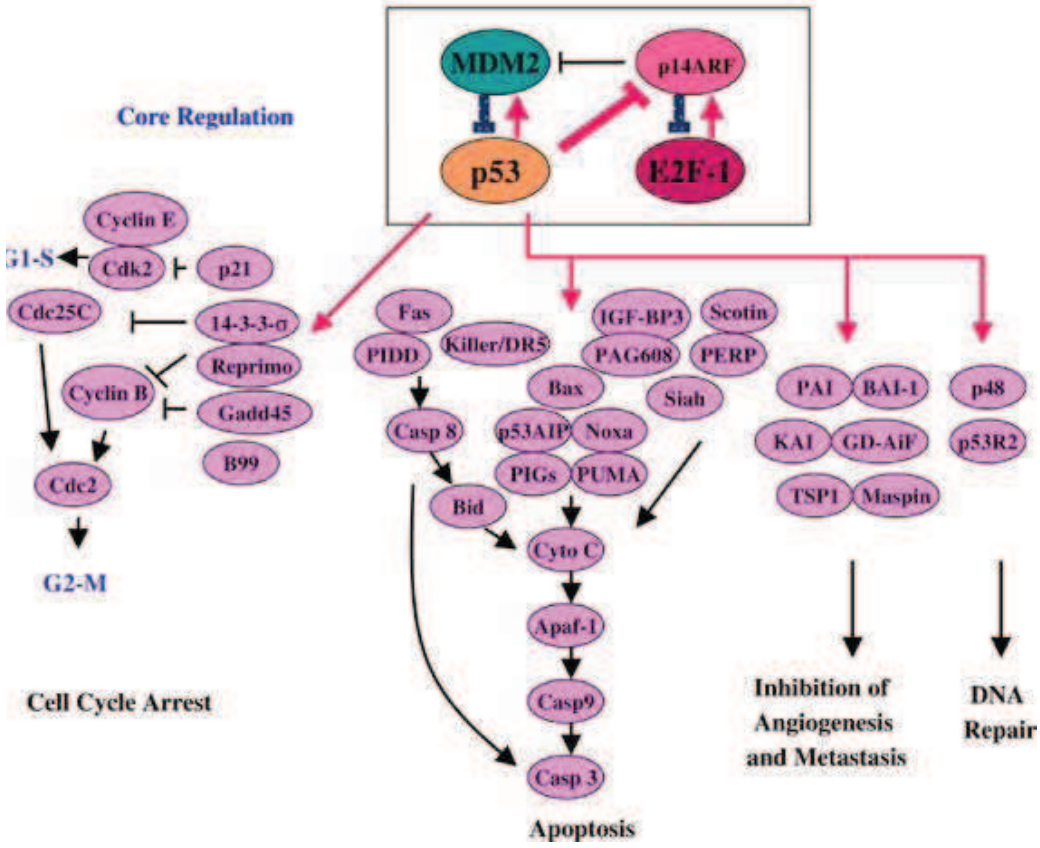
Small Ubiquitin-like Modifier – SUMO – and Neural Precursor Cell Expressed, Developmentally Down-regulated 8 – NEDD8 – are ubiquitin-like proteins covalently linked to lysines. SUMO is both involved in p53 activation and repression whereas neddylation seems to be not completely defined but promotes p53 activity (Xirodimas *et al.* 2004; Abida *et al.* 2007).

A.IV. p53 pathways

There are many different outcomes to p53 induction characterized by hundreds of described responsive genes to p53 – for a review of characterized responsive elements of target genes and Figure 7 (Harris and Levine 2005; Riley *et al.* 2008). However the authors considered this list only as a partial p53 transcriptome and many p53 target genes are still to discover. Cawley *et al.* and Wei *et al.* identified between 500 and 1600 potential target genes by ChIP based techniques, which let us consider a Dantesque task to integrate these new regulations to the previously existing p53 database (Cawley *et al.* 2004; Wei *et al.* 2006).

Once p53 is activated and bound to DNA it is able to activate transcription by different main events: The direct recruitment of the transcription machinery proteins: the TATA Binding Protein – TBP – and TBP Associated Factors – TAF – 6, 9 which will attract the RNA polymerase to the DNA, the recruitment of Chromatin Remodeling Factors – CRF – like the SWItch/Sucrose Non Fermentable – SWI/SNF – complex or Histone Acetyltransferase – HAT – or Methyltransferases – MT – like mentioned earlier and the recruitment of components of the mediator complex.

Figure 7. Target genes of p53 and their related pathways.



p53 is able to induce many different responses through its target genes. It acts by its transcriptional activity and its ability to bind key protein effectors. From (Harris and Levine 2005).

Contrariwise to the so-called p53 transcriptional activity, p53 repress significant number of genes. This can occur by three different means: binding site overlap of other transcription activators, squelching of transcriptional activators or recruitment of Histone Deacetyltransferases – HDACs – Serum Response Factors – SRFs – HATs and MTs (Laptenko and Prives 2006). Indeed p53 transcription repression does not necessarily require a p53 RE on the contrary to transcriptional activation. Finally, p53 is able to repress overall transcription by inhibiting the RNA Pol I, II and III promoters, by direct or indirect means (Zhai and Comai 2000; Gridasova and Henry 2005).

This report will quickly overview principal pathways involved but also will described more precisely how p53 can affect angiogenesis.

A.IV.1. Survival pathways

In vivo and *in vitro* studies showed that cells devoid of p53 expression are more sensitive to DNA damage than cells expressing wild type p53 (Xu *et al.* 1995; Wahl *et al.* 1996; Cote *et al.* 1997). This puzzling aspect of p53 has been later solved by

uncovering the ability of p53 to favour DNA repair. This with the ability of p53 to stop cell cycle progression constitutes the classical survival pathway.

A.IV.1.a.Cell cycle

Proliferating cells undergo a series of four different phases – G1, S, G2 and mitosis – composing the cell cycle during which DNA is duplicated – S phase – and cells divide – mitosis. DNA and cells integrity is checked after S phase and mitosis at cell cycle arrest – G2 and G1, respectively. The different steps of the cell cycle are strongly regulated by Cyclin Dependent Kinases at three checkpoints: G1/S checkpoint, S checkpoint and G2/M checkpoint. p53 is induced upon abnormal progression through the cell cycle.

- G1/S transition checkpoint:

Under normal growth conditions, progression through G1 is promoted by D-type and E-type cyclins and their associated Cyclin Dependent Kinases – cdk2, cdk4, and cdk6. If DNA is damaged, Chk1 and ChK2 kinases are activated and transfer the signal to p53, which induces p21WAF1/CIP1, a Cdk inhibitor (Herold S *et al.* 2002). p21 sustains G1 arrest by inhibiting cdk2 and cdk4 activities. While p21 is the primary regulator of p53-mediated G1 arrest, other p53 target genes are also involved in maintaining G1 arrest, such as B-cell Translocation Gene 2 protein – BTG2 – and Growth Arrest and DNA Damage 45 – GADD45.

- S phase checkpoints:

When S phase takes place, DNA alterations occur. However this phenomenon is in part physiological as replication is imperfect. At least two checkpoints protect the cell during this vulnerable time: the intra-S checkpoint and the replication checkpoint. The intra-S phase checkpoint is activated when DNA damage occurs during S phase. Likewise G1/S checkpoint, ATR-ATRIP heterodimer and ATM are recruited at site of DNA lesions, activates Chk1-Chk2 and these kinases induce a halt in DNA replication by blocking the binding of Cdc45 to chromatin that normally enhances DNA pol-α into pre-replication complexes (Lukas *et al.* 2004). Although p53 is phosphorylated and stabilized by ATR and Chk1, p53 does not appear to have a major contribution to S phase arrest.

- G2/M checkpoint:

The G2/M checkpoint is essential for cell fate because it controls normal progression to mitosis. It mainly prevents segregation of damaged chromosomes. In

order to sustain G2/M arrest, Cdc2-cyclinB activity must be inhibited. In this respect, p53 regulates many target genes that play critical roles during G2/M arrest. For example, p53 induces p21 which blocks G2/M progression by binding the Cdc2-cyclinB complex and preventing the phosphorylation of Cdc2 at Thr161 by CDK-Activating kinase – CAK – (Smits *et al.* 2000). p53 also induces 14-3-3σ which blocks entry into mitosis by promoting Wee1 kinase activity, and sequestering Cdc25C phosphatase in the cytoplasm. Moreover, other p53 targets such as GADD45, BTG2, REPRIMO, have been implicated in the maintenance of the G2/M checkpoint.

A.IV.1.b DNA repair

As there are multiple damaging events that can cause DNA to be altered, and multiple consequences in term of DNA alteration, DNA repair involves a broad range of elaborated mechanisms to recover DNA integrity. Five principal pathways are required: the Nucleotide Excision Repair – NER – the Base-Excision Repair – BER – and the Mismatch Repair – MMR – that consecutive of SSBs and the Non-Homologous End-Joining – NHEJ – and the Homologous Recombination – HR – that follows DSBs. p53 inhibits HR on the contrary to the four others – for complete reviews see (Sengupta and Harris 2005; Helton and Chen 2007). Interestingly p53 is a general activator of DNA repair by increasing the chromatin accessibility to DNA Repair involved proteins (Rubbi and Milner 2003).

A.IV.2. Cell death pathways

The two best described pathways in which p53 is involved are cell cycle arrest and apoptosis. It is widely accepted that induction of cell death pathways are consequent to a threshold in p53 activation following stress signals.

A.IV.2.a. Apoptosis

As well as cell cycle arrest, apoptosis implies the induction of a large set of genes that are responsive to p53 induction: p53 Upregulated Modulator of Apoptosis – PUMA – Noxa – latin for *damage* – Bcl-2-Associated X protein – BAX. Apoptosis can be divided by two sub-pathways both involving p53: the intrinsic and the extrinsic pathways.

The intrinsic pathway is induced by transcription activation of BH3-only proapoptotic like PUMA, NOXA and BH3 Interacting Domain Death Agonist – BID – B-cell Lymphoma 2 – BCL-2 – family proteins like BAX protein or Actin Filament-

Associated Protein-1 – AFAP-1. PUMA and NOXA sequester the antiapoptotic associated proteins BCL-2 or BCL-XL and thus release the pro-apoptotic proteins BAX and BAK. BAX and BAK can oligomerize and form a pore in the mitochondrial outer membrane releasing among others the cytochrome c from interstitial membrane compartment. Once in the cytoplasm, cytochrome c forms a complex with several monomers of APAF-1 and procaspase-9 to constitute the apoptosome. Activated by ATP, the apoptosome cleaves and activate procaspase-3, 6 and 7 leading to DNA fragmentation and generation of apoptotic bodies – for a review see (Fridman and Lowe 2003; Danial and Korsmeyer 2004).

In addition to its transcriptional activity, p53 uses transcription independent activation for its ability to bind directly BCL-2 and BCL-XL proteins in mitochondria.

The extrinsic apoptosis pathway involves the activation of transmembrane receptors, the well named Death Receptors – DR4 or DR5. Their intracellular death domain clusterizes with other death receptors and recruits the adaptator Fas-Associated Death Domain – FADD – and procaspase 8 and 10 – initiator caspases – forming the Death Inducting Signaling Complex – DISC. Procaspases 8 and 10 are cleaved and activated which in turn activate the caspase 3, 6 and 7. Although the main ligand of these DR is the p53 independent ligand Apo2L/TRAIL, p53 can induce the transcription of the Fas ligand gene TNF Ligand Superfamily member 6 – TNFSF6 – the DR 5 and Fas/CD95 death receptor genes. Therefore, p53 sensitize the extrinsic pathway, enhancing the susceptibility to the environment.

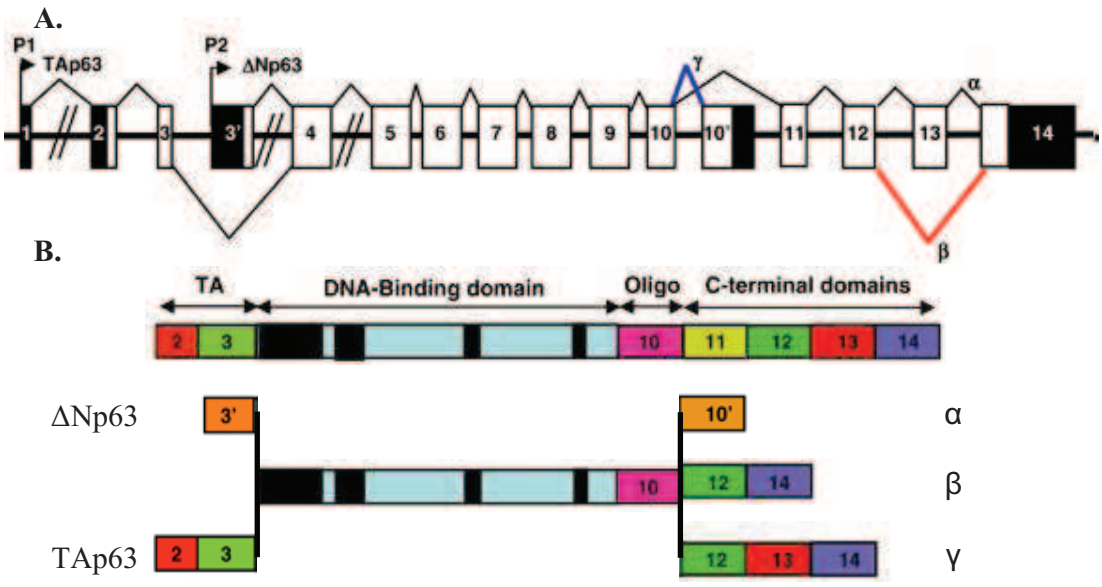
A.V. p53 family

In addition to the p53 gene, two other genes with a similar structure and phylogenetically conserved have been discovered: p63 and p73 genes (Kaghad *et al.* 1997; Yang *et al.* 1998). In invertebrates only one p53 gene exists, suggesting a triplication of the gene during evolution (Yang *et al.* 2002). Despite the genetic redundancy between p53, p63 and p73 sequences, each one of these gene have specific patterns of target genes. The profile of p63 and p73 isoform expression has been predominant to reconsider the p53 isoform expression.

A.V.1. p63 and p73 family genes.

A.V.1.a. p63 gene.

Figure 8. Structure of p63 gene and its isoforms.



Scheme of the human p63 gene – A - and proteins – B. From (Murray-Zmijewski *et al.* 2006).

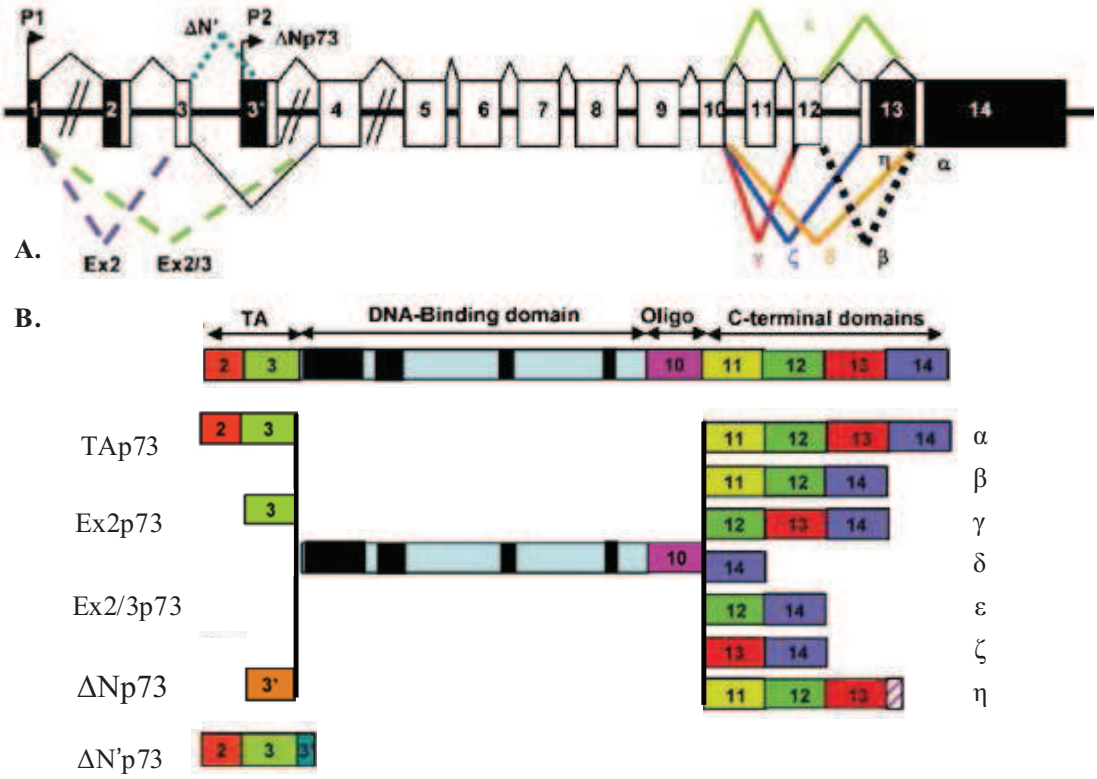
In 1998 Yang described the p63 gene which bears strong homology with p53 and p73 genes – Figure 8 (Yang *et al.* 1998). Osada *et al.* discovered it in the same time and named it p51 but only p63 lasted (Osada *et al.* 1998). This last group found p63 able to transactivates p21 and induce cell arrest and apoptosis. Present in human and mouse tissues, p63 encodes multiples isotypes that can interplay with p53 modulating its transcriptional activity. N-truncated isoforms seem to be predominant in epithelial cells. Human p63 consists in three different C-terminal splicings – α/β/γ – and an internal promoter in the intron three – respectively TAp63 and ΔNp63 for full length and truncated p63 isoforms. If TAp63 isoforms have transactivating activity, ΔNp63α which is the most expressed isoform between truncated spliced isoforms, contains a specific short transactivating domain in N-terminal which can inhibit cell proliferation, induce cell death, and up-regulate GADD45, p21 and others (Helton *et al.* 2006).

A.V.1.b. p73 gene.

In 1997, Kaghad discovered a p53 related gene: p73 – Figure 9 (Kaghad *et al.* 1997). The p73 locus is also frequently deleted – but barely mutated – in cancer and particularly in neuroblastoma. They showed that p73 transactivates some p53 target gene such as p21 and is able to bind p53. The pattern of isoform is conserved in p73 gene, although the number of resulting isoforms is much bigger, giving a more complex map. The p73 gene expresses at least 7 alternatively spliced C-terminal isoforms – α, β,

γ, δ, ε, ζ and η – and at least 4 alternatively spliced N-terminal isoforms, which contain different parts of the TAD. Altogether, the p73 gene expresses at least 35 mRNA variants which can encode theoretically 29 different p73 protein isoforms. An additional sterile alpha motif – SAM – within the C-terminal region, which is not present in p53, is also required for p63 and p73 transcription factor activity. SAM-containing-proteins are involved in regulation of development and in differentiation. The exact molecular partners are consequences of this regulation are largely reviewed in Zawacka-Pankau *et al.* (Zawacka-Pankau *et al.* 2010).

Figure 9. Structure of p73 gene and its isoforms.



Scheme of the human p73 gene – A - and proteins – B. From (Murray-Zmijewski *et al.* 2006).

A.V.2. p53 isoforms

Until 2005 the structure of p53 gene was quite simple. In 1993 Chow identified an alternative splicing in the exon 9 by PCR amplification in a lymphoblastic leukemia cell line. This splicing was resulting from a deletion upstream the donor site to the exon 9, creating a novel donor site (Chow *et al.* 1993). Many mutations and deletions modify significantly p53 gene structure leading to expression of abnormally spliced p53 mRNAs in human cancers.

The first alternative splicing of p53 in human was identified in 1987. Matlashewski *et al.* showed an alternative splicing at the acceptor site in intron 2

resulting in an altered 5' end coding region named EIIp53 (Matlashewski *et al.* 1987). They did not however associate it with the expression of an alternative isoform.

Indeed the first isoform has been described in 1996, Flaman *et al.* showed that p53 gene was submitted to an alternative splicing in the exon 9 in lymphoblastic cells (Flaman *et al.* 1996). The resulting new mRNA contained an additional 133 bp exon which is translated into an isoform lacking the p53 tetramerization domain and failed to bind DNA. This isoform was upregulated in quiescent cells.

In 2002 Yin *et al.* found that in response to DNA damage treatment, mdm2 induces the expression of an isoform of p53 of 47 kDa (Yin *et al.* 2002). This isoform called p53/47 comes from the alternative initiation of translation in the exon 4 does not contain the mdm2 binding site and thus is not sensitive to mdm2 proteasomal induction. In the same year Courtois *et al.* described the same isoform – called DNp53 – and found the same characteristic regarding mdm2 binding and showed that this isoform negatively regulates p53 transcriptional activities (Courtois *et al.* 2002). They also demonstrated that DNp53 is accumulating in serum starved conditions and phase S but not in response to DNA damage, suggesting a cell cycle regulator of p53. Another group further characterized this isoform demonstrating it shuttles between cytoplasm and nucleus during cell cycle (Ghosh *et al.* 2004).

In 2005 our group discovered and characterized nine isoforms of p53 (Bourdon *et al.* 2005). The presence of alternative splicings was already pointed out in the precedent studies but also in the studies concerning p63 and p73. However no internal promoter was found yet in human p53. In the same year, Chen *et al.* in collaboration with our group showed that the zebrafish p53 gene contained an internal promoter allowing the expression of the Δ113p53 isoforms and possessed the same gene structure (Chen *et al.* 2005). The work of our group revealed the presence by mRNA Capped specific RT-PCR of several new isoforms which structure was present as well in *Drosophila*. In fact, p53 has a similar gene structure to its homologs p63 and p73. It has two alternative splicings in the exon 9 that allow the expression of α, β and γ isoforms, and an internal promoter in intron 4 that defines the expression of the Δ133p53 isoforms. Importantly p53 isoforms are differentially expressed in human normal tissues. Finally we confirmed the presence of an alternative initiation of translation site in exon 4, 40 codons after the first start codon. This latter isoforms named Δ40p53 isoforms are the one characterized by Courtois and Ghosh groups – named p53/47 or DNp53. These 9 nine isoforms are schematically represented in Figure 3. Rohaly at al.

described another isoform resulting from skipping exon 8 and expressing the Δp53 isoform (Rohaly *et al.* 2005). This isoform is also expressed in normal human tissue and activated following UV treatment. They also showed Δp53 was able to induce p21 and 14-3-3σ but not MDM2 nor BAX and did not tetramerize with p53. However the results found by this group were not confirmed by others (Chan and Poon 2007; Garcia-Alai *et al.* 2008). We did not detect it neither in normal tissues, nor in couples of tumour tissues, as well as other groups (Anensen *et al.* 2006; Goldschneider *et al.* 2006; Boldrup *et al.* 2007; Bourdon 2007)

A.V.2.a. Δ40p53 isoforms

Since its discovery Δ40p53 is the subject of much interest because it has been shown to modulate significantly and depending strongly on cell cycle and specific stresses. Indeed p53/47 – or Δ40p53 – translation is controlled by an alternative mechanism of initiation of translation called IRES (Candeias *et al.* 2006; Yang *et al.* 2006). Through cytotoxic agents such as platinum derived complex LA-12 this isoform is able to induce apoptosis (Hrstka *et al.* 2008). They also showed that this isoform can enhance p53 transcriptional activity – to p21 for instance – by forming tetramer insensitive to MDM2 binding (Powell *et al.* 2008). Finally they showed that Δ40p53 is translationally activated by endoplasmic reticulum stress and induces G2 cell cycle arrest (Bourougaa *et al.* 2010). An interesting review focuses on the role of translation mechanisms on p53 and its isoform Δ40p53 (Grover *et al.* 2009)

A.V.2.b. Full length p53 spliced isoforms.

This paragraph will mainly focus on the p53β isoform as little is still known about the p53γ. It is maybe due to its lack of stability, as we observed experimentally – unpublished data.

Our group has shown that endogenous p53β bind preferentially BAX promoter and poorly MDM2 promoter and has partial pro-apoptotic activity. p53β is able also to form complexes with p53 and modulates p53 activity. Further more, we raised a specific antibody specific to p53β – KJC8. Another study by our collaborators Fujita *et al.* confirmed this first work (Fujita *et al.* 2009). It showed the implication of p53β in senescence as its expression level increased with the number of cell passages – at replicative senescence – in normal human fibroblast strains – MRC-5 and WI-38. When

overexpressed, p53β inhibited cell proliferation and induced cell senescence probably by increasing p21 and miR34 expression.

A.V.2.c. Δ133p53 isoforms

Δ133p53 isoforms are expressed as three different splices – Δ133p53α, Δ133p53β and Δ133p53γ. Δ133p53α is expressed in a wide range of normal human tissues, while Δ133p53β and Δ133p53γ are expressed in few normal human tissues (Bourdon *et al.* 2005). Thus when I will term Δ133p53 I will refer mainly to Δ133p53α although in most cases segregating between them is still not technically possible. One important aspect of Δ133p53 has been revealed by our work in 2005: overexpression of Δ133p53 inhibits p53 mediated apoptosis after co-transfection in a p53 null cell line. So this protein is considered as negative dominant to p53. Consistently our collaborators Fujita *et al.* showed that Δ133p53 is able to inhibit senescence and is downmodulated in senescent human fibroblasts (Fujita *et al.* 2009). Δ113p53, the counterpart in zebrafish of Δ133p53 in humans, bears interestingly activities that could help us to understand Δ133p53 ones. Indeed, Δ113p53 depletion by morpholinos injection in zebrafish embryos sensitizes embryos to p53 dependent apoptosis after ionizing radiation (Chen *et al.* 2009). More importantly this work reinforced our first hypothesis of a dominant negative effect and also suggested a role as a modulator of p53 response. They showed that Δ113p53 has its own intrinsic activity and transactivates BCL-XL gene in a mutant p53 context.

A.V.2.d. p53 isoforms in tumourigenesis

One thing is to characterize p53 isoform in cell line models and *in vitro* but revealing the importance of these isoforms in the development of cancer is truly essential. In breast cancer, p53 gene is mutated only in 25% of the cases; suggesting that p53 is inactivated by alternative pathways. Consequently, we first identified strong differences in the p53 isoform expression profile between normal breast tissue and tumour tissue. We showed that there was a loss of p53β expression – 60% of the cases – and an overexpression of Δ133p53 forms – 40% of the cases – in breast cancer. These results strongly suggest that abnormal expression of p53 isoforms explain the low percentage of p53 mutation in breast cancers.

Anensen *et al.* analysed the modifications in p53 expression profile in acute myeloid leukemia – AML – and showed a strong expression of an unidentified p53 C-

terminal truncated isoform – more important than the full length p53 – in AML (Anensen *et al.* 2006). After 4 hours treatment of chemotherapy the ratio between p53 and this isoform increased drastically.

Boldrup *et al.* investigated the p53 expression profile in human head and neck tumours (Boldrup *et al.* 2007). They found strong variations between tumour samples, non tumour samples surrounding the tumour and non tumour samples. Marabese *et al.* analysed by quantitative PCR the levels of expression of Δ133p53, Δ40p53 and p53 in phase I and phase III ovarian cancer. They first found comparable levels between each set of isoforms and no differences between phase I and phase III cancers.

In melanoma cells and primary tumours, Avery-Kiejda *et al.* investigated the p53 expression profile and found expression of p53β and Δ40p53 in tumour cells but not in normal cells (Avery-Kiejda *et al.* 2008). They also found a variation of Δ133p53 expression between normal and tumour cell lines, and a general change in p53 expression profile suggesting an involvement of p53 isoforms in melanoma malignancy.

Renal cell carcinoma – RCC – has been also the subject of investigations. Song *et al.* analysed quite exhaustively the p53 expression profile in normal and tumour samples and cells (Song *et al.* 2009). They found that RCC and normal renal cells expressed all p53 isoforms apart from the Δ133p53β which is not expressed in normal cells. They also found an overexpression of p53β in cancer suggesting a role for this protein.

Fujita et al, besides their approach in senescence modulation by Δ133p53 and p53β isoforms, analyzed the expression of these proteins in colon adenomas and carcinomas and correlated a senescent phenotype with increased Δ133p53 and decreased p53β, indicating the overcome of this latter phenotype.

The few reports we have just looked upon show us two main informations regarding p53 expression profile in cancer – For a summary of p53 isoform expression, see Table 1. First the profile can vary depending on the tumour type and stage. Second, the scientific tools available to investigate the p53 isoforms are not sufficient enough to specifically analyse p53 isoforms expression in relation to clinical markers and clinical outcome. For instance by nested PCR we are able to identify specifically the presence of each p53 isoforms. However, it would be much more informative to quantify specifically the expression level of each isoform. Nevertheless, we can definitely

confirm the importance of the p53 isoforms regarding the present clinical data in several cancer tissues.

Likewise, Chen *et al.* investigated the p53 isoforms activities during embryogenesis in zebrafish. They determine that p53 isoforms play an important role during development by inhibiting p53 mediated apoptosis. These findings suggest that p53 isoforms have biological activities beyond carcinogenesis. Therefore, Prives and Manfredi had the best word to summarise in what the discovery of these isoforms had contributed to the entire p53 world: “more sleepless nights ahead” (Prives and Manfredi 2005).

Table 1. Expression of p53 and its isoforms in normal tissues and cancers.

From (Bourdon *et al.* 2005)	p53			Δ133p53				
Tissue	α	β	γ	α	β	γ	Cancer	Reference
Colon	+	+	+	+	+	+	Overexpression of Δ133p53α and downregulation of p53β in transition from adenoma to carcinoma	(Fujita *et al.* 2009)
Bone Marrow	+	+	+	+	+	+		
Testis	+	+	-	+	+	+		
Kidney	+	+	+	+	-	+	Overexpression of p53β and Expression of Δ133p53β	(Song *et al.* 2009)
Brain	+	-	+	+	-	-		
Fetal brain	+	+	-	+	+	+		
Heart	+	+	+	+	-	-		
Salivary gland	+	+	+	+	-	-		
Spinal chord	+	-	-	+	-	+		
Lung	+	-	-	+	-	-		
Spleen	+	+	-	+	-	-		
Placenta	+	ND	ND	+	ND	ND		
Adrenal gland	+	ND	ND	+	ND	ND		
Thymus	+	ND	ND	+	ND	ND		
Uterus	+	+	+	-	-	+		
Breast	+	+	+	-	-	-	Expression of Δ133p53α and loss of p53β	(Bourdon *et al.* 2005)
Prostate	+	-	+	-	-	+		
Squeletal muscle	+	-	+	-	-	+		
Fetal liver	ND(+)	-	-	ND	-	-		
Intestine	ND(+)	+	+	ND	-	+		
Tyroid gland	ND(+)	+	+	ND	-	+		
Stomac	ND(+)	+	+	ND	-	+		
Normal tissue from head and neck	+	+	+/-	+/-	-	-	Higher statistical p53γ expression	(Boldrup *et al.* 2007)

B. Angiogenesis

Angiogenesis is a physiological process involving the growth of new blood vessels –BVs – from a pre-existing network. In contrast vasculogenesis describes the formation of new BVs from the differentiation of circulating endothelial progenitors known as angioblasts. Vasculogenesis mainly occurs in the embryo resulting in the formation of the primordial systemic complex of vasculature which has to be effective before the start of heartbeats. On the contrary angiogenic processes only occur after vasculogenesis and consist in remodeling of vasculature to readapt the blood circulation system to the constant and enormous changes that will occur until maturity.

B.I. Circulatory system

The circulatory system is a unique organ system composed by the cardiovascular system which includes the heart, arteries, capillaries and veins, and the lymphatic system which carries lymphatic vessels, nodes and the thoracic duct.

B.I.1. Structure and functions of the blood vessels.

The cardiovascular system is a typical asymmetric organ devoted to supply effectively and continuously the tissues with oxygen and nutrients throughout the body and to collect the metabolic waste like carbon dioxide. The arteries are the efferent BVs from the heart whereas the veins are the afferent ones.

B.I.1.a. Arteries and Veins

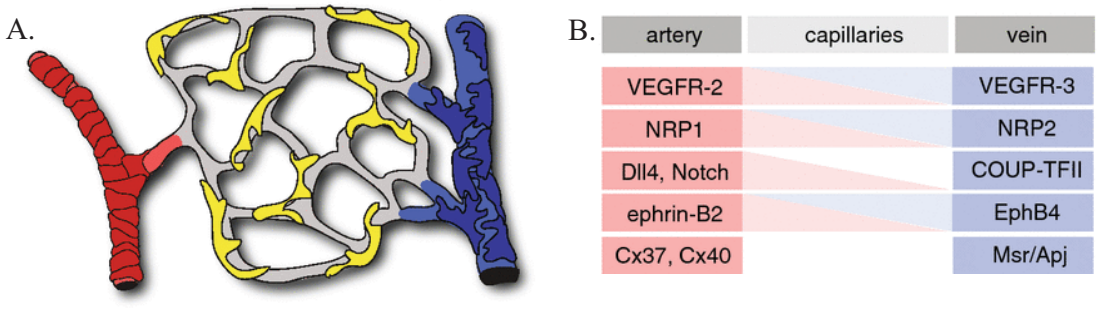
This implies that arteries must be extremely resistant to the variations of blood pressure and flow induced by the contraction of the heart. To overcome this pressure arteries adjacent to the heart possess a thick wall containing elastin – 40% – and Smooth Muscle Cells – SMC – in the tunica media. Consequently to the decrease of blood pressure arteries – elastic arteries – give rise to thinner walls devoid of elastin – muscular arteries –arterioles and finally capillaries. They make a bridge between arteries and veins. Similar in structure, veins are however characterized by thinner walls containing only few elastin and SMCs. In order to avoid backflow due to gravity and lack of pressure, veins contain bileafvalves in the lumen.

Since arteries and veins are subject to different constraints and differ in their functions, molecular pathways have been elucidated to understand the underlying

mechanism of arterio-vascular identification – Figure 10. Principally, arterial cells specifically express the gap junction proteins Connexin-37 and 40 – Cx37 and Cx40 – components of the Notch pathway – Dll4 – Vascular Endothelial Growth Factor – VEGF – co-receptor Neuropilin-1 – NRP1. On the other hand, venous cells specifically – or at least predominantly – express the co-receptor Neuropilin-2 – NRP2 – receptor VEGFR3, COUP Transcription Factor 2 – COUP-TFII – that negatively regulates the Notch pathway, and the Apelin receptor (Rocha and Adams 2009).

Although arteries and veins show some structural, molecular and functional differences, they are both composed of three tunicae containing endothelial cells – ECs – SMCs and fibroblasts – Figure 11.

Figure 10. Arteries, veins, capillaries and thier specific markers.



A. Arteries are represented in red, veins in blue and EC of the capillary bed in grey. Pericytes partially covering the capillaries are colored in yellow. Structure of SMCs in blue and red show a strong difference in morphology. B. Markers of arteries and veins can either be specifically expressed in one compartiment or be differentially expressed. From (Rocha and Adams 2009).

B.I.1.a.α. The tunica intima and endothelial cells.

This is the internal and thinner tunica. It is composed of a monolayer of ECs recovered by a vascular basal membrane – subendothelial layer.

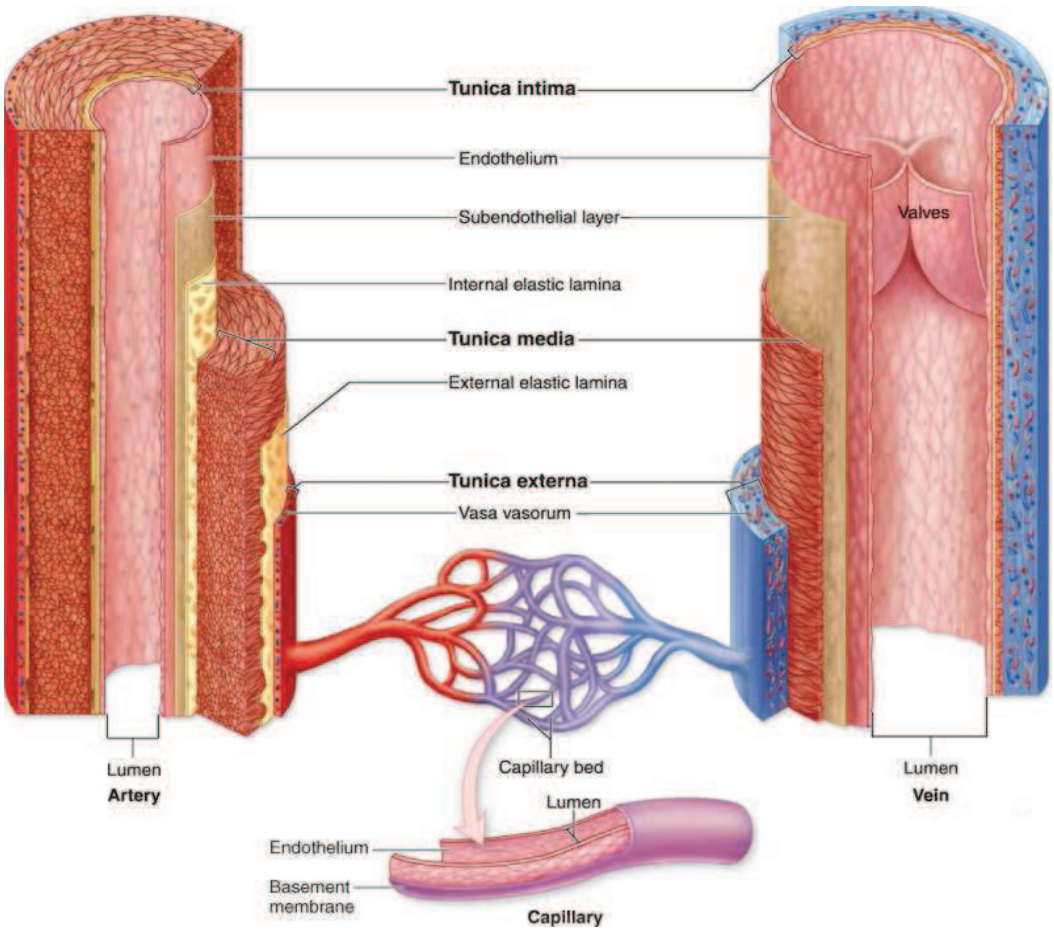
- The endothelial cell layer is polarized. On the contrary to the apical side in contact with the blood stream, the basal side in contact with the basal membrane is pro-thrombogenic and secretes procoagulating molecules: Collagen IV and the Von Hippel Factor – pVHL. Tumour cells are thought to enhance a pro-coagulation stage that favours metastasis in certain types of tumours (Maragoudakis *et al.* 2000).

- The endothelium regulates the arterial vasomotricity by vasodilatating molecules like endothelin or thromboxane A2 or vasoconstricting factors like NO. These components act upon the internal parts of the artery and principally to the SMCs.

- It allows blood cells and components to penetrate and reach the internal layers of the artery, to ensure the nutrition and the defence of these cells.

The endothelium has a strategic localisation in vessel wall due to its functions of communication between the blood and the circulatory system and the other parts of the vessel wall. Finally, the endothelium maintains the blood vessel in a vasodilatation, non proliferating state and block inflammatory cells to adhere. Upon activation ECs induce vasoconstriction, blood platelet and monocytes adhesion and proliferation of SMCs.

Figure 11. Structure and organization of Arteries, Veins and Capillaries



This schema drows differences and similarities between arteries and veins. Both bear sequently a layer of ECs, a basal membrane, the tunica media consistuted of SMCs and elastin and the tunica externa – or adventitia – constituted of fibroblasts in majority. From (Tortora and Grabowski 2003).

B.I.1.a.β. The tunica media and the smooth muscle cells.

The media is constituted by concentric layers of SMCs forming the thick internal part of the artery, lined by an internal and an external elastin layer. Each layer is surrounded by an extracellular matrix containing fibrous proteins and – in elastic arteries – elastin. Arterioles display only one layer of SMCs. While elastic arteries dilate and passively shrink on each blood pulse from the heart, muscular arteries function to respond to their afferent tissue demands.

B.I.1.a.γ. The tunica adventitia and the fibroblasts.

Although the adventitia is composed of a majority of fibroblasts within a connective tissue rich in collagen and elastic fibers, many different cell types are encountered in this external layer. Recent experimental evidence has identified fetal and adult arterial and venous vessel walls as niches for stem and progenitor cells such as endothelial and smooth muscle progenitor cells, hematopoietic stem cells and mesenchymal stem cells (Tilki *et al.* 2009) and adipocytes. The adventitia and the media are irrigated by the *vasa vasorum.*

B.I.1.b. The capillary network

This latter only exhibits ECs, a basal membrane and sparse pericytes partially covering the endothelial cell vessel. Pericytes and SMCs can be distinguished by several markers like α-actin, desmin and Platelet Derived Growth Factors – PDGFR-β – (Gerhardt and Betsholtz 2003). Pericytes are closely linked to the endothelial basal membrane and to several ECs by gap and "peg-socket" junctions and synergize EC communication (Caruso *et al.* 2009). Besides to capillaries, pericytes are located in the pre-capillar arterioles, the veinules gathering points and the post-capillar veinules.

The second population of cells in capillaries exhibits also different morphology depending on the organ. Three different types of endothelial cell walls can be encountered:

- Continuous capillaries constituted by firm lining of strands of tight junctions with rare intercellular clefts. They are found in skeletal muscles, lungs, gonads, and skin. A more sophisticated version includes specific transporters for glucose, amino acids, and other substances. These latter capillaries are found in central nervous system and form the brain-blood-barrier.

- Fenestrated capillaries: ECs of endocrine glands, pancreas, intestine, kidney glomeruli and liver sinusoids contain small – 60-70 nm in diameter – but densely clustered pore-like openings permitting only small molecules and limited amount of proteins to diffuse.

- Sinusoidal or discontinuous capillaries are present for instance in the liver, spleen, bone marrow and the lymph nodes. They are constituted by larger pores – 30-40 μm in diameter – that allow red and white blood cells to cross over as well as some serum proteins. This structure helps the transfer of metabolites between blood vessels and tissues.

B.I.2. The lymphatic system

B.I.2.a. Role of the lymphatic network

In contrast to the blood system the lymphatic system is a one way system that drains the excess of liquids – lymph – antigens and macromolecules like fatty acids from every tissue in the whole body to the lymphatic tissue and nodes, to finally be rejected into the left and right subclavian veins. This draining is an important task that ensures oncotic equilibrium of the interstitial space. The lymphocytes are also transported in the lymphatic system and produced by the lymphoid organs like the thymus, the spleen and the bone marrow. The lymph is a vector for immune cells to amplify their response in the lymph nodes and then get back to the blood system. The lymphatic system is primal target for metastasis dissemination because first of its intrinsic collecting function and also by the leaky structure of the blind-ended lymphatic endothelial capillaries.

The Lymphatic ECs – LECs – constitute a monolayer of ECs that are not wrapped by any pericytes nor SMCs and does scarcely or not have basement membrane. The structure features of the lymphatic capillaries i.e. discontinuous junctions and interjunctional gaps makes it highly permeable and enables leukocytes entry (Tammela and Alitalo 2010).

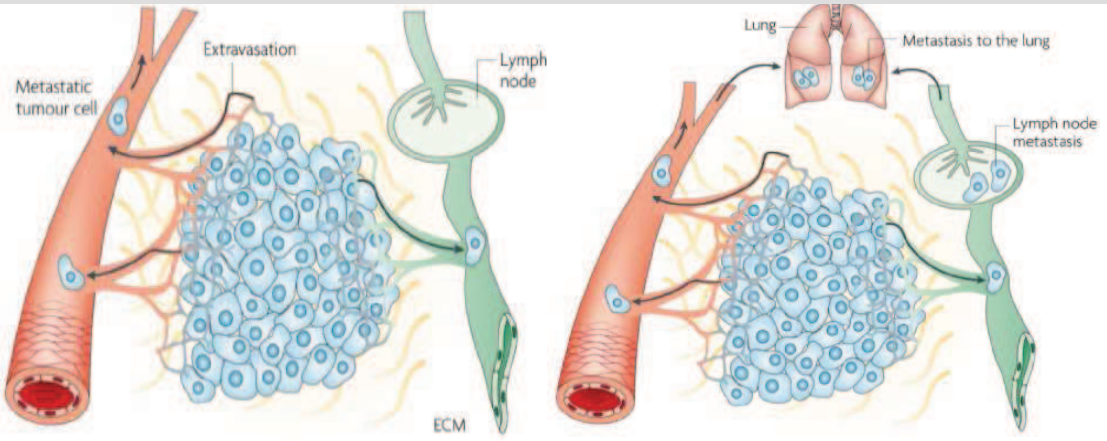
As well as blood vessel veins, lymphatic vessels contains bileaflat intraluminal valves to prevent lymphatic backflow. Besides the monolayer of ECs and the basal membrane, collecting lymphatic vessels possess a layer of SMCs and also an adventitia that contains connective tissue and fibroblasts. On the contrary of the common belief, lymphatic vessels demonstrate regular, strong, fast phasic contractions implying both lymphatic pumping and flow resistance (Muthuchamy and Zawieja 2008).

B.I.2.b. Tumour Lymphangiogenesis

Lymphangiogenesis, the growth of new lymph vessels, is involved in the adult in tissue inflammation, wound healing, and tumour metastasis – Figure 12. Tumour cells disseminate first through the lymphatic system *via* sentinel lymph nodes in various types of cancer like breast, colon and prostate carcinomas. It is thought that even prior to tumour metastasis dissemination to the whole body, tumour cells engage their remodeling and their settlement in the sentinel lymph nodes (Qian CN *et al.* 2006). It is currently discussed to know whether this step of dissemination is just a hallmark of

tumour transit towards the next organs or if it plays a role in amplification for systemic metastasis (Joyce and Pollard 2009).

Figure 12. Dissemination and metastasis through blood and lymphatic vessels.



While blood vessels expand their network towards the tumour, lymphatic vessels also develop new vessels that will help the draining of the microenvironment and the dissemination of cancer cells. Indeed in priority to invasion of blood vessels, cancer cells evade the tumour niche by the lymph and can cause lymph node metastasis and secondly to other organs. From (Avraamides *et al.* 2008).

B.II. Physiologic and pathologic angiogenesis.

In healthy adults, angiogenesis occurs in physiological changes such as the hairy skin and the female reproductive system, e.g. in the ovary, in the uterus, in the placenta and development of the mammary gland during pregnancy. Otherwise, vascular development in the adult is linked to tissue repair and clinical disorders like wound healing and tumour growth, rheumatoid arthritis, diabetic retinopathy and psoriasis (Folkman 1971; Folkman 1995; Plendl *et al.* 1999; Plendl 2000; Tamanini and De Ambrogi 2004; Nagy *et al.* 2007; Schenone *et al.* 2007). The formation of these BVs is mastered by a sophisticated balance of numerous pro and antiangiogenic molecules that non solely inhibit or activate angiogenesis but prime a set of various processes that will lead to a mature and efficient new blood organization, or lead to the regression of the overall vasculature.

B.II.1 The angiogenic switch

In normal conditions, the maintenance of steady blood and lymphatic vessels is provided by the balance of proangiogenic and antiangiogenic factors released by the microenvironment. In fact, antiangiogenic factors are thought to be responsible of the quiescent state of the ECs whereas proangiogenic factors can activate ECs. In some pathological conditions like in cancer, the overall balance is modified – irreversibly – to

obtain an induction of blood vessel formation. Many growth factors, cytokines and other extracellular matrix components like the VEGF, Fibroblast Growth Factor 2 – FGF-2 – angiopoïetins, Platelet Derived Growth Factor - PDGF - and Transforming growth factor – TGFβ - are involved in this process. They and others will be discussed further in this essay. Besides these molecular modulations, metabolic stresses – hypoxia, hypoglycemia or acidosis for instance – mechanical stress and shear stress or inflammatory response can induce an angiogenic switch (Carmeliet and Jain 2000).

Yet this angiogenic switch is a paramount step of tumour progression and transformation. If a tumour raises a certain size – 1 to 2 mm3 – and does not have sufficient oxygen and nutrient supply, it becomes quiescent or necrotic and even apoptotic (Holmgren *et al.* 1995; Parangi *et al.* 1996). In fact, some of these tumours – also called micrometastases because of their size – that are not malignant enter either a dormant state in which their mutagenic capacity is blocked or have a division/death ratio that does not permit the tumour to grow. These tumours can be present all over the body and do not induce cancer until they escape dormancy by stimuli like genetic mutations, hypoxia and other metabolic stresses, mechanical stress, and the immune/inflammatory response (Carmeliet and Jain 2000). Unfortunately, surgical removal of the primary tumour in breast is often correlated with shortening of dormancy and promotes the growth of micrometastasis (Demicheli *et al.* 2008). These data are reinforced by the fact that only a local burst of angiogenic factor is able to irreversibly switch on the dormant tumour leading to tumour growth and metastasis (Indraccolo S *et al.* 2006).

B.II.2. Steps of blood vessel formation.

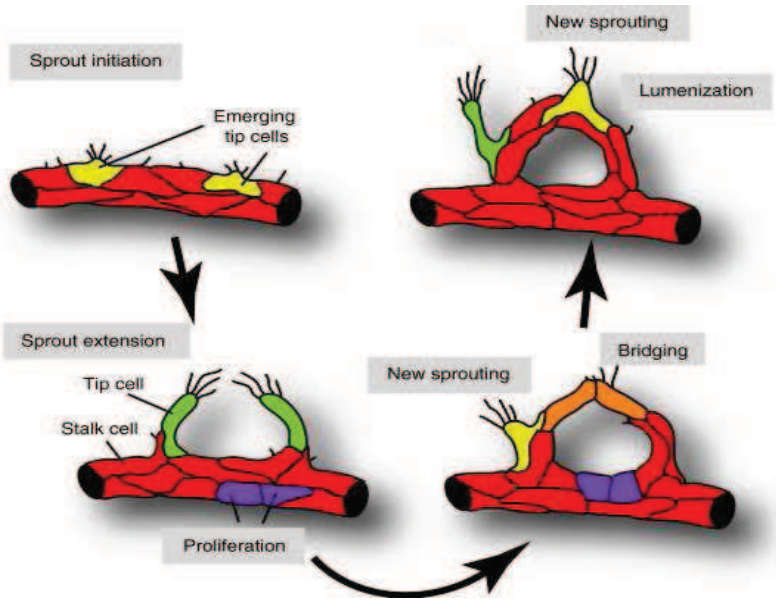
Various mechanisms involve the remodeling and the neovascularization of BVs commanded by the microenvironment in response to physiological activities or pathological stresses, followed by a step of maturation – also called resolution step - and recruitment of peripheral blood cells such as smooth muscle cells Here are presented the common mechanisms observed in angiogenesis.

B.II.2.a. Sprouting angiogenesis

Sprouting angiogenesis is described as the prevalent event and best characterized event of angiogenesis – Figure 13 (Gordon *et al.* 2009). The release of proangiogenic factors like VEGF into the surrounding stroma causes the activation of the remodeling

the vasculature prior to the formation of new BVs. Indeed the multi-layered structure of blood vessels is disrupted by the detachment of pericytes and SMCs from the ECs, dilatation of blood vessel wall, increase of vascular permeability by the redistribution of adhesion molecules like Platelet Endothelial Cell Adhesion Molecule-1 – PECAM-1 – and Vascular Endothelial-cadherin – VE-cadherin – and loosening of the extracellular matrix. Proteases embedded in the extra cellular matrix – ECM – such as Urokinase plasminogen activator – uPA – heparanases, metalloproteases or growth factors like FGF-2 are released and now able to degrade the basal membrane of the endothelial layer and create permissive intercellular space for circulating ECs. In the same time, plasmatic proteins – Angiopoïetin-1, FGF-2, and VEGF – guide EC through out the interstitial matrix. One of them is activated and will guide the growing sprout by making filopodia and lengthen itself in the direction of the stimulus: it is the capillary tip cell. It migrates and forms a cone with a layer of EC underneath – the stalk cells – the whole forming a cavity and a lumen. In physiological angiogenesis, selection of tip cells and hierarchy between tip and stalk cells is achieved by a cunning Notch-DLL4 regulatory pathway and by ECs crosstalks. On the contrary tumours models cause Notch-DLL4 inactivation and induce excessive sprouting and branching of BVs (Suchting *et al.* 2007). Maturation of the blood vessel is achieved by mural cells and synthesis of the ECM.

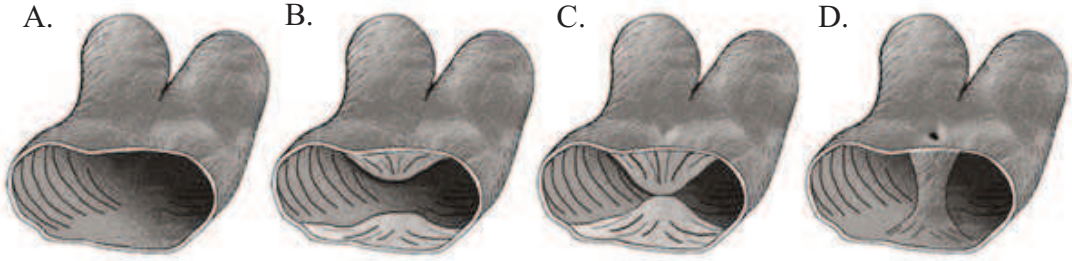
Figure 13. Steps of sprouting angiogenesis



Upon angiogenic stimuli *via* proangiogenic factors like VEGF and FGF-2 certain ECs activate their sprouting program and lead the formation of new blood vessels. These "tip cells" extravasate, form filopodia to allow motility, lengthen them and pull other ECs with them. These are the stalk cells and have the ability to keep vessel integrity and expend to form the lumen of the new vessel. Tip cells can meet and form a bridge, finalize the new blood vessel while new tip cells arise. From (Adams and Eichmann 2010).

B.II.2.b. Intussusception.

Figure 14. Steps of intussusceptive angiogenesis



3D diagram illustrates steps of intussusceptive angiogenesis. Following stimuli like shear stress or mechanical pressure, blood vessels begin vessel division called intussusception – A. Opposite walls approach each other *via* protrusions of ECs towards the lumen – B. When each side meets they tighten the contact – C, which is followed by central perforations, where fibroblasts, pericytes and connective proteins such as collagens will lock and finalize the separation of vessels parts. From (Djonov *et al.* 2003).

Intussusceptive angiogenesis is an alternative angiogenic phenomenon firstly described by scanning electron microscopy – Figure 14 (Caduff *et al.* 1986). Contrarily to sprouting angiogenesis it does not requires a large number of EC divisions and permits a fast remodeling of the environment. Preexisting vessels split into two new vessels by formation of a transvascular tissue pillar into the lumen of the vessel. This process can be divided into four steps: Opposite ECs make a "kissing contact", reorganize to form a cavity between the EC layers, surrounding pericytes and SMCs migrate and cover the newly interstitial pillar. Finally the pillar extends its own diameter and allows separating the new vessels.

Intussusception in tumour angiogenesis could explain the rapid BV remodeling (Djonov *et al.* 2001). This phenomenon relies on molecular events that are still not clearly understood but it has been noticed that shear stress – sensed by PECAM-1 – and blood pressure are key stimuli triggering intussusception.

B.II.2.c. Recruitment of endothelial progenitor cells – EPC.

Since the finding in 1997 of well characterized EPC in the blood stream and their implication in angiogenesis (Asahara *et al.* 1997), a lot of controversy is ongoing about the importance or predominance of the recruitment of EPC in relation to sprouting angiogenesis. A core aspect for the assessment of progenitor's recruitment is the current availability of specific markers for each specific study and subtype of cells (Tilki *et al.* 2009).

The recruitment and the mobilization of EPCs from the bone marrow – compared to other origins – seem to be the prevalent and best described mechanism. In summary bone marrow endothelial progenitors are activated by VEGF, Placental Growth Factor – PlGF – Stroma Cell-Derived Factor-1 – SDF-1 – and angiopoïetin-1 (Hillen and Griffioen 2007). The recruitment and integration of EPCs implicates chemoattraction, anchoring, and homing within the angiogenic site, integration into the new vessel and differentiation into mature ECs.

B.III. Tumour Angiogenesis.

The growth of solid tumours is extremely dependent on nutrient supplies from the environment: they only grow until a size of 100 µm3 – around 4 to 7 layers of cells, depending of the type, from the closest blood vessel – using efficiently passive transport of nutrients and oxygen between cells. Beyond this size, tumours must start to trigger gradients of angiogenic stimuli pulling BVs towards the tumour spot. Without this new inflow, tumours can not overcome 1 to 2 mm3.

The principal difference between a physiologic angiogenic process – including wound healing – and tumour angiogenesis is the tight regulation of each factor that permit the elaboration of a functional, structured and matured BVs network. In contrast, the vasculature pointing at the tumour is under constant and overwhelming induction, conferring it a tortuous, extorted and dilated phenotype with dead ends. This vasculature is known to be poorly efficient, leaky and hemorrhagic (Bergers and Benjamin 2003).

B.III.1. Tumour-related angiogenesis

The mechanisms described below perfectly underline the plasticity of some types of cancer cells in their capacity to resist and adapt to the lack of oxygen and nutrient supply. They are even able to redefine their nature and functions in order to keep on going or to resume the metastasis process despite the extremely challenging microenvironment. This rises up deep concerns on the strategy of antiangiogenic therapies and their efficiency in aggressive tumour models. More than all, strong knowledge and smart profiling of the tumour from each patient will be one solution for successful treatment.

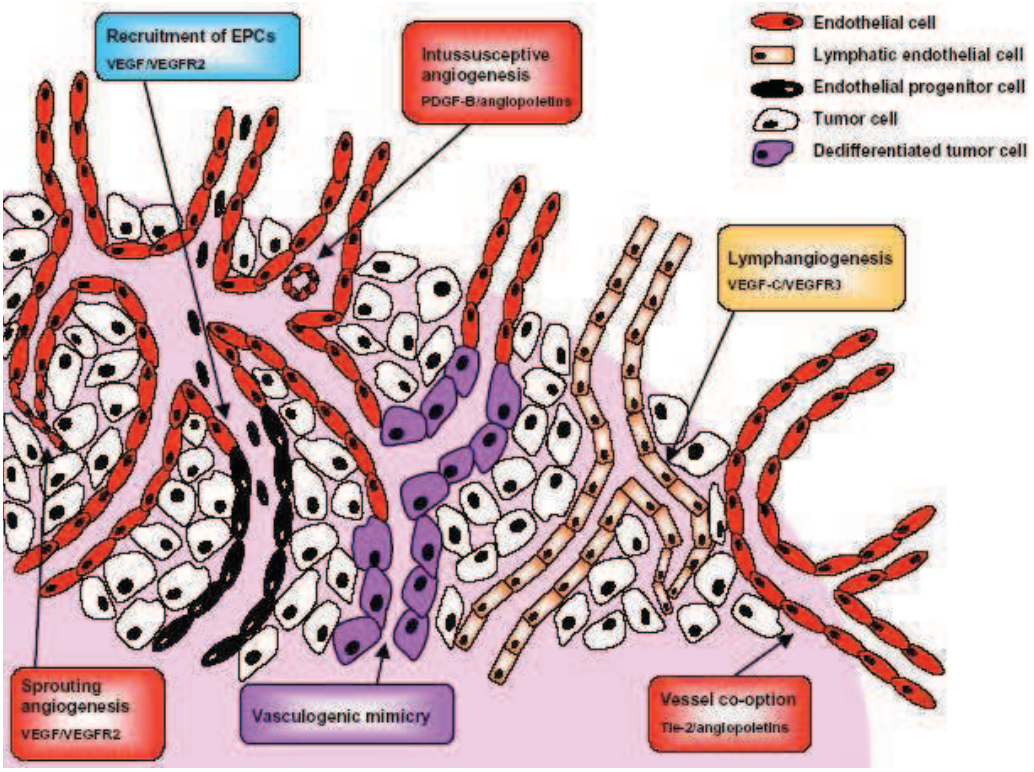
B.III.1.a. Vasculogenic mimicry – VM.

Firstly discovered in 1999 by Maniotis *et al.*, it describes the capability of tumours cells to dedifferentiate and to form tube-like structures without a clear endothelial phenotype. Revealed by Periodic Acid-Shiff's reagent – PAS – staining and negative CD34 ECs staining, VM has also been observed in many cancers (Shirakawa *et al.* 2002; Sharma N *et al.* 2002; Sood *et al.* 2000; Sun *et al.* 2004; El Hallani *et al.* 2010). Typical aggressive tumours show a reversion to a more undifferentiated embryonic-like phenotype expressing endothelial associated genes such as VE-cadherin, Ephrin A2 and tissue factor pathway inhibitors, tyrosine kinase receptor 1, neuropilin 1, E-selectin and endoglin (Hendrix *et al.* 2001; Hendrix *et al.* 2003). However the mechanism leading to formation of endothelial like tubes is still not understood.

VM was shown to be an alternative mean in aggressive tumours to escape insufficient blood supply and to be stimulated by antiangiogenic strategies. Interestingly, canonical growth factors like VEGF, FGF-2 or Transforming Growth Factor β – TGF-β – are not implicated in this phenomenon as they do not induce VM in non aggressive melanoma cell lines OCM-1A and UMEL-1 (Maniotis AJ *et al.* 1999).

B.III.1.b. Cooption.

Figure 15. Tumour angiogenesis mechanisms.



Principal mechanisms involved in tumour angiogenesis. If sprouting angiogenesis is considered as the main event, other mechanisms take part considerably in this phenomenon, depending on the tumour type. From (Hillen and Griffioen)

In highly vascularized tissues, tumour grows and cancer cells migrate next to blood vessels in order to be correctly supplied (Holash et al. 1999). By switching from the Ang-1 to the Ang-2/Tie-2 pathway tumour cells can induce regression of blood vessels and reduced blood perfusion. This mechanism induces necrosis of blood vessels that allows the tumour to grow furthermore, and hypoxia that will exacerbate the production of angiogenic factors like VEGF by tumour cells and facilitate the formation of new vessels. This type of angiogenesis underlies the heterogeneity of tumour samples : in the center the tumour is devoid of nutrient supply and is often necrotic (Holash *et al.* 1999) whereas the periphery of the tumour is responsible for the angiogenic activity.

B.III.1.b. Mosaic vessels.

Firstly described in 2000, Chang *et al.* baptized mosaic BVs as heterogenic BVs constituted by EC and tumour cells activated by natural killer cells, in colon carcinoma xenografts (Chang *et al.* 2000). It resulted in their study that 15% of vessels were mosaic vessels, forming 4% of the whole vasculature. However, the presence of tumour cells within the vascular network seems to be transient as it is consequent of tumour cell intravasation in the lumen that will stay briefly in the capillary wall. FGF-2 and VEGF-A induced tumour cells intravasation (Folkman J. 2001)

B.IV. Molecular signaling in tumour angiogenesis

B.IV.1. VEGFs

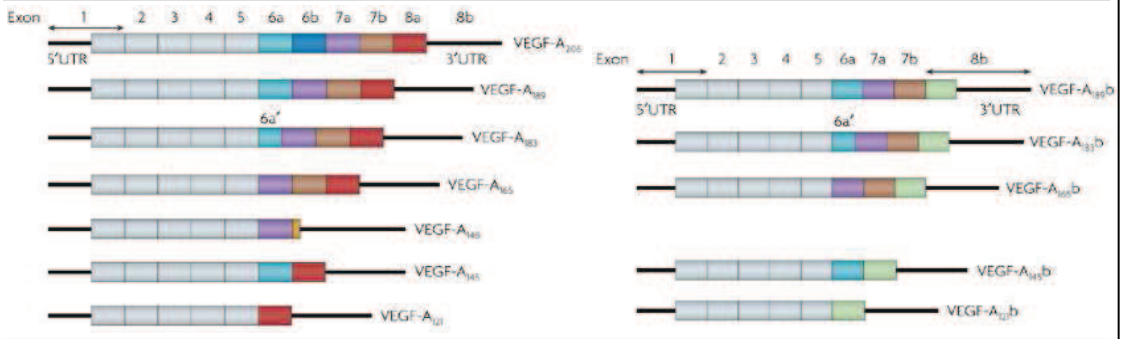
The VEGF family is composed of five mammalian cytokines, VEGF-A, VEGF-B, VEGF-C, VEGF-D, and Placenta Growth Factor – PIGF – plus two viral VEGFs: VEGF-E and VEGF-F. VEGFs are homodimeric disulfide bonded glycoproteins that bind Heparan Sulphate Proteoglycan – HSPG – and three Tyrosine Kinase Receptors – TKR – VEGFR-1, VEGFR-2 and VEGFR-3 plus two co-receptors lacking the intracellular tyrosine kinase activity, NRP-1 and NRP-2 and an alternatively spliced VEGFR-1, soluble receptor VEGFR-1. To activate the transduction pathway VEGF dimers binds to TKRs that will dimerize and autophosphorylate. TKRs activation can induce angiogenesis, vasculogenesis or lymphangiogenesis. Each VEGF ligand has its own specificity with its respective TKR: VEGF-A, prominent amongst VEGF ligands and thus also called VEGF, induces angiogenesis and vasculogenesis through VEGR-2, which is ECs specific, whereas VEGF-C mainly induces lymphangiogenesis through VEGR-3 (Neufeld *et al.* 1999; Grothey and Galanis 2009).

B.IV.1.a. VEGF-A

The splicing of the eight exons of the VEGF-A gene resulted in several isoforms with different angiogenic potentials. Alternative splicing of exon 6 – a and b – 7, 8 and proteolytic cleavage rise to anti and proangiogenic isoforms varying in their ability to bind HSPG and consequently to be present in ECM – Figure 16. In basal conditions overall VEGF-A expression is dominated by VEGF-A_{xxx}b isoforms whereas their expression strongly decrease in pathological context (Harper and Bates 2008). VEGF-A_{xxx}b isoforms compete equally with VEGF-A_{xxx} ones in the binding of VEGFRs but does not induce sufficient transduction activation and rather induce degradation of is VEGFRs.

In human VEGF 165, 121 and VEGF 189 mainly constitute VEGF-A_{xxx} isoforms and the ability to bind HSPG distinguishes them. From highly diffusible to completely bound are VEGF121, 165 and 189. The interest of sequestered VEGFs is to have a pull of available factors *in situ* that will be quickly functionally maturated either by uPA or plasmin is response to various aggressions.

Figure 16. VEGF-A and VEGF-A_{xxx} isoforms.



All VEGF-A and VEGF-A_{xxx} isoforms are synthesized from alternative splicings of exon 6, 7 and 8 resulting in 12 isoforms. The most studied isoform up to now are the VEGF-A_{189}, VEFGF-A_{165} and VEGF-A_{121}, due to their proangiogenic potential and their differences in secretion, VEGF-A_{121} being the most secreted. VEGF-A_{XXX} isoforms have opposite effects and generally counteract the VEGF-A isoforms. Ratio of them is proposed to be an important determinant for angiogenic switch. From (Harper and Bates 2008)

B.IV.1.a.α. VEGF-A expression

VEGF-A is constitutively expressed at low levels in normal adult tissues whereas it is recurrently overexpressed in several cancer types and constitutes one of the strongest diagnostic markers for evaluation of tumour gravity. This overexpression is the result of an adaptive behavior of the tumour in a stringent environment. Indeed, hypoxia is one important setting for VEGF overexpression. Under low level of oxygen,

pVHL no longer targets HIF-1α to proteasomal degradation and induces its dimerization with HIF-1β counterpart, which will translocate to the nucleus and activate transcription of various genes such as VEGF-A. In a second hand, hypoxia favours VEGF mRNA stability probably by interaction with HuR (Ciais *et al.* 2004; Ido *et al.* 2008). Low pH, another characteristic of tumour microenvironment, induces VEGF-A independently of HIF-1α (Fukumura D *et al.* 2001).

In tumours VEGF-A is expressed in high levels even if there is no patent hypoxia. Actually this upregulation can be achieved by several mechanisms such oncogene activation, tumour suppressor inactivation or by induction from growth factors. For instance src ,c-myc or Ras activation are known to increase VEGF-A mRNA and protein, similarly as pVHL inactivation, p53 and p73 mutation (Nagy *et al.* 2007).

In addition several cytokines have shown to be able to induce VEGF expression like PDGF, Tumour Necrosis Factor α – TNF-α – TGF-β, IGF and Interleukin 6 – IL-6 – and IL-1β (reviewed in Dvorak HF. 2002). All these cytokines are directly or indirectly regulated by the NF-κB and Activated Protein-1 – AP-1 (Pradeep *et al.* 2005).

B.IV.1.a.β. VEGF-A pathways.

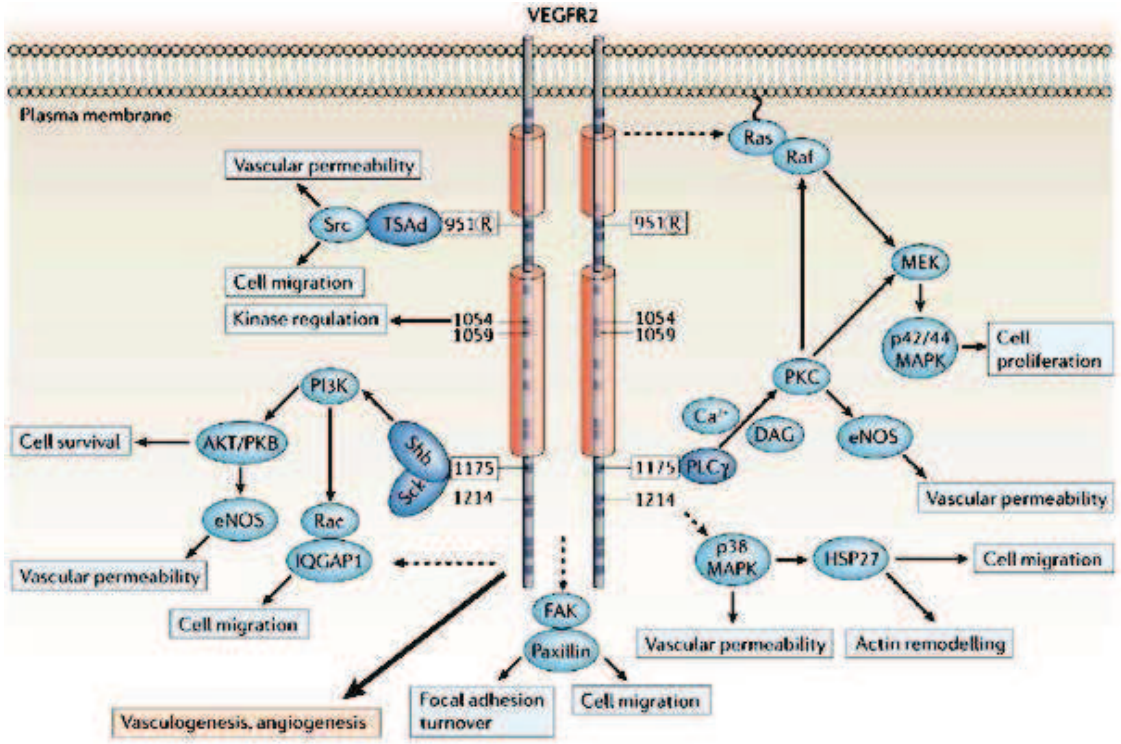
Due to the VEGF-A pivotal role in angiogenesis this report will only restrict its observation to a general view. VEGF-A triggers a cascade of signaling events: VEGFR2 Receptor dimerization and autophosphorylation, downstream phosphorylation of various proteins such as PKC, PLC-γ and PI3K. Detailed overview of the pathway involved is discussed in the following reviews – Figure 18 (Olsson *et al.* 2006; Rahimi 2006; Shibuya 2008).

If VEGF-A binds equally to VEGFR-1 and -2, VEGFR-1 has a poor tyrosine kinase activity which indicates that VEGFR-1 could act by ligand trapping or heterodimerization. NRP-1 and -2 enhance VEGF-A binding to VEGFR-2.

B.IV.1.a.γ. Biological Effects of VEGF-A.

VEGF-A has been first described in 1983 as a vascular permeabilizing factor from tumour ascites fluids (Senger *et al.* 1983). Actually as an immediate action, VEGF-A induces vasodilatation through NO synthesis, microvascular permeability and extravasation of plasmatic proteins (Dvorak *et al.* 1979; Horowitz *et al.* 1997).

Figure 18. VEGF-A signalling



Upon dimerization and activation on VEGF-Receptor – here VEGF-R2 – multiple pathways are triggered such as the MAP kinase pahtwaypathway, the phospholipase ? - PLC? - pathway, the focal adhesion kinase – FAK – pathway, the Src and the Phosphatidyl inositol 3 phosphate pathway – PI3K. This allows couples of cellular responses which activate the cell to an angiogenic phenotype. Numbers represent the phosphorylation site of the tyrosine. From (Olsson *et al.* 2006).

Src: sarcoma; TSAd: T-cell-specific adapter protein; Shb: Src homology 2 domain containing adaptor protein B; AKT/PKB: serine/threonine protein kinase B; eNOS: endothelial NO synthase; Rac: Rho GTPase protein; IQGAP1: IQ motif containing GTPase activating protein 1; MAPK: Mitogen activating protein kinase; MEK: MAPK Kinase; PKC: Protein kinase C; DAG: Diacylglycerol; HSP27: Heat shock protein 27

All other activities of VEGF are consequent of gene expression regulation. Indeed, VEGF is able to activate migration and proliferation of arterial and venal ECs and lymphatic cells *in vitro.* In this purpose it regulates proteins related to blood clotting and fibrinolysis i.e. Tissue Factor – TF – uPA, tissue Plasminogen Activator – tPA – Plasminogen Activator Inhibitor-1 – PAI-1 – etc, proteases i.e. Matrix Metalloproteases – MMPs – glucose transporters, NO synthase and numerous mitogens. It is also able to inhibit cell death *via* apoptosis *in vitro* and *in vivo* by several antiapoptotic factors i.e. BCL-2, survivin, X-linked Inhibitor of Apoptosis – XIAP (Gerber *et al.* 1999). VEGF-A acts upon cell to cell recognition and cell to matrix interaction regulating adhesion molecules like E-selectin, Inter-Cellular Adhesion Molecule – ICAM-1 – and Vascular Cell Adhesion Molecule – VCAM (Olsson *et al.* 2006; Rahimi 2006; Shibuya 2008).

Besides its role in angiogenesis VEGF can have some relevant role in inflammation as it can recruit monocytes and induce the formation of granulocytes and

macrophages (Clauss *et al.* 1990; Broxmeyer *et al.* 1995). In return, these cells as well as lymphocytes T produce proangiogenic factors like PDGF and MMP-9 that will contribute maturation of newly formed vessels (Freeman *et al.* 1995; Rafii *et al.* 2002).

B.IV.1.b. Other VEGF members

- VEGF-B exists as two isoforms of 167 and 186 a.a. from a single gene. VEGF-B 167 and 186 do not bind VEGFR2 but rather VEGFR1 and VEGF-B 167 binds also to HSPG. VEGF-B is expressed embryonically, in some adult tissues and in cancer. However VEGF-B does not support strong activity apart from cardiac function and uPA proteolytic activation (Olofsson *et al.* 1998; Roskoski 2007).
- VEGF-C is highly expressed in adult heart, ovary, placenta, skeletal muscle, and small intestine, but is present also in kidney, lung, pancreas, prostate, spleen, and testis (Joukov *et al.* 1996). By its binding to VEGFR-3 and in a weaker manner to VEGFR-2 VEGF-C is a potent activator of lymphangiogenesis in embryo and in tumour associated lymphangiogenesis. VEGF-C is required for the migration and survival of lymphatic ECs. VEGF-C is expressed by a significant fraction of human tumours including those of breast, cervix, colon, lung, prostate and stomach (Siegfried *et al.* 2003; Onogawa *et al.* 2005; Su *et al.* 2006). VEGF-C KO mice die at embryonic state E15.5 to E17.5 due to impairment of lymphatic vascular development.

Also involved in lymphangiogenesis and binding to VEGFR3 and VEFGR2, VEGF-D is less described maybe because effects on VEGF-D KO mice are normal despite its upregulation in several types of cancers.

- Placental growth factor is expressed as four isoforms primarily in the placenta but also in diverse human tissues. PlGF null mice are viable and fertile despite diminished vascularization of the retina and the *corpus luteum*. PlGF enhances expression of VEGF.

B.IV.2. FGF-2

FGF-2 – a.k.a. bFGF – is a member of the super family of FGFs which consists of 23 members for 18 ligands – FGF11 to 14 do not function as ligands and FGF15 gene does not exist. FGF family contains numerous members divided in seven subfamilies but particularly two members, FGF-1 and FGF-2 are well investigated and show relevant activities. All FGF members have affinity to HSPG which is essential to the subsequential binding of the TKR type FGFRs. Ligands of FGF family encompass

various activities on different types of cells, such as mitogens, induce cell migration, cell survival and differentiation. They also act upon different times in development, adult physiology and pathology, from embryo steps to wound healing and cancer promotion.

B.IV.2.a. FGF-2 Biosynthesis

B.IV.a.α. FGF-2 gene expression

FGF-2 is transcripted from a single gene FGF2 in chromosome 4 and contains only three exons (Mergia *et al.* 1986; Shibata *et al.* 1991). FGF-2 promoter has only one +1 transcription site, no TATA or CAAT box, five Sp1 binding sites in the 5' of exon 1 and one AP1 site in position -243. A large number of transcription factors regulate the FGF-2 promoter activity. Homeobox protein Hox-B7 – HOXB7 – IL-1, and EGF, PDGF and FGF-2 itself through Early Grown Related protein – EGR-1 – upregulate FGF-2 transcription whereas cell confluency and interferon α and β inhibit FGF-2 transcription (Biesiada *et al.* 1996; Care *et al.* 1996; Moffett *et al.* 1996; Wang *et al.* 1997). Additionally Regulator of FGF-2 Transcription – RTF – and p53 have been identified as inhibitors of FGF-2 transcription (Ueba *et al.* 1994; Ueba *et al.* 1999). Interestingly thalidomide, a potent and controversial drug that bears antiangiogenic activity binds to the G-rich FGF-2 promoter as well as the coding region giving another step to understanding of thalidomide modes of activity (Mei and Wu 2008). Finally a small mRNA from antisense transcription of FGF2 gene is able to destabilize FGF-2 RNA (Li and Murphy 2000).

B.IV.2.a.β. FGF-2 translation regulation

Matured FGF-2 RNA is constituted by a 5'UTR of 484 nt particularly rich in GC bases, a 468 nt coding region and an extremely long 3'UTR of 5823 nt which contains several polyadenylation sites, destabilization sequences and translation activation (Prats *et al.* 1989; Touriol *et al.* 1999; Touriol *et al.* 2000).

The 5'UTR of FGF-2 RNA is extremely rich in GC bases which makes it highly structured. Indeed it contains *cis* regulatory elements called IRES involved in alternative translation initiation which drives translation from four different non canonical codons CUG and a canonical AUG upstream the IRES driving cap-dependent translation (Prats *et al.* 1992).

Firstly described in virus, mammalian IRESs are now well described in multiple factors involved in different IRESs such as C-Myc, the chaperone Bacterial Intravenous Protein – BiP – HIF-1α VEGF-A, FGF-1 and others – for a detailed list of mammalian IRES database: http://ifr31w3.toulouse.inserm.fr/iresdatabase/ or http://iresite.org/ (Nanbru *et al.* 1997; Kim *et al.* 2002; Lang *et al.* 2002; Martineau *et al.* 2004). These specific structures give fundamental advantage in some selective stimuli because IRES dependent translation is not sensitive to stress related translation blockade. If no consensus sequences between IRESs have been identified, some general features are sometimes observable.

The FGF-2 IRES is one of particular importance amongst IRESs. Indeed FGF-2 IRES activation is tissue-specific and condition dependant such as hyperglycemia, heat shock, hypoxia or oxidative stress (Vagner *et al.* 1996; Creancier *et al.* 2000; Teshima-Kondo *et al.* 2004; Conte *et al.* 2008). FGF-2 IRES activity is also involved in brain development and testis maturation (Gonzalez-Herrera *et al.* 2006; Audigier *et al.* 2008). Cellular factors can bind to IRESs and regulate internal ribosome entry – they are called IRES Trans Acting Factors – like Heteronuclear Ribonucleoprotein A1 – hnRNPA1 – and p53 (Galy *et al.* 2001; Galy *et al.* 2001; Bonnal *et al.* 2005).

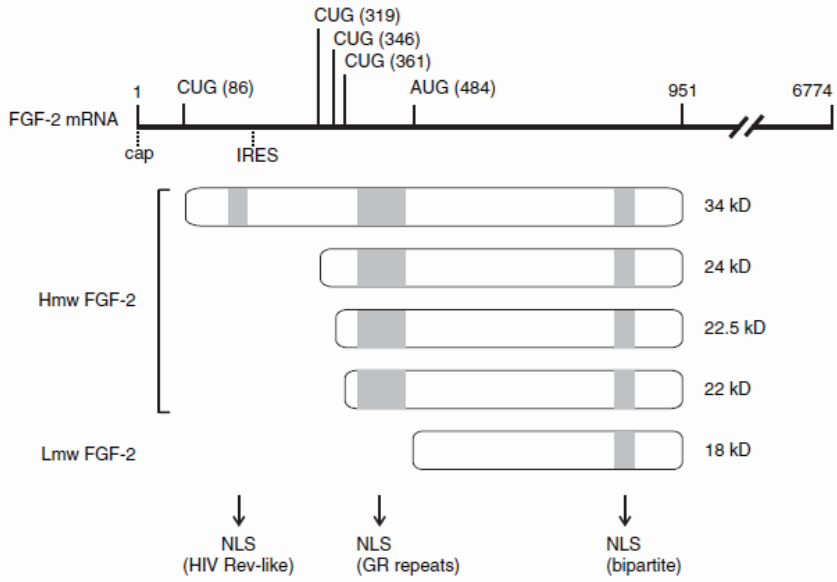
B.IV.2.b. FGF-2 isoforms

Due to five start codons in humans in one mRNA, FGF-2 is able to produce five different isoforms of 18 – a.k.a. LMW form for low molecular weight form – 22, 22.5, 24 and 34 kDa – HMW for High Molecular Weight – Figure 19 (Florkiewicz and Sommer 1989; Prats *et al.* 1989). These differences in size come with differences in function, localization and fate. The 18 kDa isoform is localized in the cytoplasm whereas the HMW are mainly located in nucleus *in vitro*, due to the presence in these isoforms of NLS (Renko *et al.* 1990; Bugler *et al.* 1991; Arnaud *et al.* 1999). HMW isoforms expression negatively correlates with cell density in human primary fibroblasts and augments with transformation due to a modulation of the use of alternative start codons (Galy *et al.* 1999). Overexpresssing HMW isoforms induce a cell cycle arrest at G2M, by inhibition of 4E-BP1 phosphorylation in glioma cell line, and inhibited *in vivo* tumour growth (Lemiere *et al.* 2008)

Contrarily to the HMW isoforms that only are present in cytoplasm and nucleus, LMW FGF-2 isoform trafficking is extended from cytoplasmic, secreted by a non conventional RE/Golgi independent mechanism allowing it to bear paracrine or

autocrine activity, and nuclear. Indeed, following binding to its FGFR receptors, FGF-2 18 kDa can either induce signal transduction or be translocated to the nucleus and activate its mitogenic capacities through transcription induction.

Figure 19. Human FGF-2 isoforms



All 5 FGF-3 isoforms come from the same mRNA thanks to the presence of an IRES and alternative start codons. The extremely long 3'UTR contains regulatory elements for its stability. The low molecular weight isoform is the major isoform and is secreted, mean while are upregulated in cancer and are present mainly in the nucleus. Shema from (Sorensen *et al.* 2006)

B.IV.2.c. FGF-2 Molecular interactions

B.IV.2.c.α. Interactions with ECM and Receptors

Within the ECM are present a multiplicity of HSPGs that are secreted from cells, or these HSPG can be bound to the cell surface. FGFs are extremely affine to HSPGs so once in the extracellular domain they generally bind to HSPGs. This binding has two main significances: to protect FGF-2 from proteases and to constitute a stock. FGF-2 can be activated following specific protease activity like heparanases and MMPs or by binding to FGF-2 Binding Protein – FGF-2 BP (Bashkin *et al.* 1992; Czubayko *et al.* 1994). There is thus a modulation of activation FGF-2 signaling through the overall composition of HSPGs and the expression of FGF-BP.

HSPGs bound to cell membrane have a different signification as they have a role of low affinity binding receptors that will display FGF-2 to high affinity receptor FGFRs. Due to their variety, the role of HSPGs-FGF-2 complexes regarding the activation of FGF-2 signaling through FGFRs has still some controversy since perlecan activate the binding and fibroglycan and syndecan inhibit it (Aviezer *et al.* 1994). It is however commonly accepted that HSPG enhance FGF-2 binding to FGFRs by

stabilizing ligand-receptor complex (Raman *et al.* 2003). As well as VEGFRs, dimerization of FGFRs is essential to TKR activity, autophosphorylation and signal transduction.

Table 1. List of FGF members and thier related recpetors

Name	Synonyms	Signaling through high-affinity receptors
FGF-1	Acidic FGF, aFGF	FGFR-1, IIIb & IIIc; FGFR-2, IIIb & IIIc; FGFR-3, IIIb & IIIc; FGFR-4
FGF-2	Basic FGF, bFGF	FGFR-1, IIIb & IIIc; FGFR-2, IIIc; 4 FGFR-3, IIIc; FGFR-4
FGF-3	Int-2	FGFR-1, IIIb; FGFR-2, IIIb
FGF-4	kFGF, kaposi FGF, hst-1	FGFR-1, IIIc; FGFR-2, IIIc; FGFR-3, IIIc; FGFR-4
FGF-5		FGFR-1, IIIc; FGFR-2, IIIc
FGF-6	hst-2	FGFR-1, IIIc; FGFR-2, IIIc, FGFR-4
FGF-7	KGF	FGFR-2, IIIb
FGF-8	AIGF	FGFR-2, IIIc; FGFR-3, IIIc; FGFR-4
FGF-9	GAF	FGFR-2, IIIc; FGFR-3, IIIb & IIIc; FGFR-4
FGF-10	KGF-2	FGFR-1, IIIb; FGFR-2, IIIb§
FGF-16		FGFR-1, IIIc; FGFR-2, IIIb; FGFR-4
FGFs 17–		FGFR-1, IIIc; FGFR-2, IIIc; FGFR-3, IIIc; FGFR-4
FGF-20	XFGF-20	FGFR-1, IIIc; FGFR-2, IIIb;
FGF-21		FGFR-1, IIIb; FGFR-3, IIIc;
FGF-22		FGFR-2, IIIb
FGF-23		FGFR-1, IIIb; FGFR-3, IIIc; FGFR-4

List of the 22 FGF-2 members and their affilliate recpetors. From (Powers *et al.* ; Schwertfeger)

FGF Receptors constitute a family of five tyrosine kinase members – 1 to 5 – in which each receptor possesses different splicing isoforms in the N-terminal extracellular domain – Table 1. Splicing is an essential mechanism that drives the specificity of FGFR to each FGF. Together with ligand specificity, FGFRs are prone to be expressed in defined conditions and tissues, during development, proliferation and cell differentiation. For instance FGFR2b is only expressed in epithelial cells whereas FGFR2c is in mesenchymal cells. Besides these forms and similarly as VEGFRs, FGFRs exist in soluble forms and membrane bound defective TKR – like FGF-5 – that could show a dominant negative role towards FGFRs (Kim *et al.* 2001).

B.IV.2.c.β. FGF-2 transduction pathways.

Following dimerization occurs autophosphorylation in different tyrosine residues. This allows the recruitment of signaling molecules such as Src – short for sarcoma – *via* the Src Homology domain 2 – SH2. The subsequent transduction cascade involves many factors reviewed in the following journals (Schlessinger *et al.* 2000; Dailey *et al.* 2005). Four main pathways are induced downstream FGFRs phosphorylation: FGF Receptor Substrate 2 – FRS2 – Phospholipase C γ – PLCγ – Src and Phosphatydil Inositol 3 kinase – PI3K.

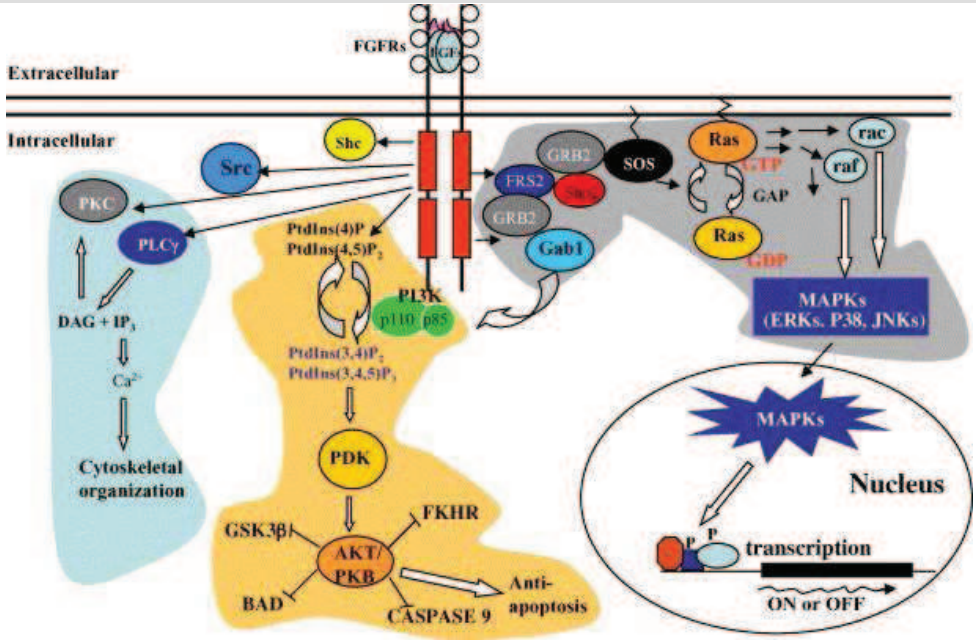
FRS2 protein, anchored thanks to a specific juxtamembrane domain in the FGFR is phosphorylated and recruits the Growth Factor Receptor Bound protein 2 – Grb2 – and Son Of Sevenless – SOS – which is a guanine exchanger. SOS activates then Ras and Mitogen Activated Protein Kinase – MAPK – pathway: Raf, Erk, p38 and JNK. This pathway transcriptionnally activates proliferation genes like c-jun, c-myc and c-fos. As a negative feedback loop FGF-2 induced MAPK inhibits FRS2, induces MAPK phosphatase 3 – MKP3 – Sprouty proteins – SPRY – and Similar Expression to FGF Genes – SEF – family members that modulate receptor signaling at several points in the signal transduction cascade (Lax *et al.* 2002; Turner and Grose 2010).

PLCγ binds FGF-2 TKR through another tyrosine phosphorylated site. This interaction leads to PLCγ activation, Phosphatidyl Inositol Biphosphate – PIP_2 – hydrolysis and generation of Diacylglycerol – DAG – and Inositol Triphosphate – IP3. These two messengers induce calcium liberation from endoplasmic reticulum and Protein Kinase C – PKC which in return induces cytoskeleton reorganization. This pathway is responsible for the migratory and cellular differentiation effects of FGF-2 (Gu *et al.* 1996; Gu and Kay 1998).

As well as PLCγ, Src phosphorylates the actin filament bound protein cortactine and may induce migration and differentiation (Tsuda *et al.* 2002).

PI3K interacts either directly with FGFRs or indirectly through GRB2-associated binding protein 1 – GAB1 – resulting in phosphorylation of 3-Phosphoinositide Dependent Protein Kinase-1 – PDK – and then AKT. PI3K activation affects a number of apoptotic factors like Glycogen synthase kinase 3 – GSK3β – Bcl-2-associated death promoter protein – BAD – which contribute to the antiapoptotic and pro-survival mechanism of FGF-2 – Figure 20 (Turner and Grose 2010).

Figure 20. FGF-2 signal transduction pathways



Principal cell types involved in FGF-2 signalling are related to angiogenesis or neuronal development. FGF-2 activate its tyrosine kinase receptors and trigger three main pathways such as PI3Kinase AKT/PKB, PLCγ/PKC and MAPKinases. From (Cao *et al.* 2008).

B.IV.2.c.γ. Intracellular protein partners.

FGF-2 is internalized through FGF TKRs and HSPG in endosomes and can translocate to the nucleus or is degraded through internalization by lysosomes. Once in the cytoplasm FGF-2 is able to interact with Casein Kinase II – CK2 – in proliferating cells which induce translocation and transcription of ribosomal genes, typoisomerases and RNA polymerase II (Bouche *et al.* 1994; Bailly *et al.* 2000). For instance nuclear FGF-2 interacts with Upstream Binding Factor – UBF – in order to engage transcription. Translokin interacts with the 18 kDa FGF-2 and is essential to its translocation into the nucleus and its mitogenic properties (Bossard *et al.* 2003). FGF-2 interacts also with Ribosomal Protein S19 – RPS19 – in the cytoplasm, Nuclear Ribosomal Protein S6 Kinase 2 – RSK2 – in S1 phase of cell cycle and ribosomal protein L6/TAXREB107, in the nucleus (Shen *et al.* 1998; Sheng *et al.* 2005). These latter interactions play also a role in the metabolism of the RNA and cell growth. HMW FGF-2 have been found to interact with the nuclear 66 kDa Subunit of Splicing Factor 3 – SF3a66 – and Survival Of Motor Neuron 1 – SMN – indicating roles in RNA splicing, as well as FGF-2 Interacting Factor – FIF – resulting in increased cell survival (Van den Berghe *et al.* 2000; Claus *et al.* 2003; Gringel *et al.* 2004).

B.IV.2.d. FGF-2 biological activities in tumour angiogenesis

Firstly identified as a murine fibroblast mitogenic factor, FGF-2 is identified as a strong inducer of angiogenesis – mediated by stroma, inflammatory and ECs or tumour cells – and cell transformation but also in embryonic development. This report will only focus on the pathological events in which FGF-2 is involved.

B.IV.2.d.α. FGF-2 and angiogenesis

FGF-2 has a broad range of actions in tumour angiogenesis. FGF-2 is involved in each step of vascular remodeling leading the synthesis of new BVs.

Indeed, it has been shown to activate ECs (Kandel *et al.* 1991). In contrast to VEGF, FGF-2 activation relies significantly more on pericytes and vascular SMCs which finally confers a much more equilibrated and efficient vasculature (Cao *et al.* 2008). Angiogenesis can be "switched on" also by the pool of FGF-2 present in the ECM.

FGF-2 has a potent role in the degradation of the ECM because it is able to activate uPA, its receptor the Urokinase receptor – UR – and some MMPs (Mignatti *et al.* 1991; Gualandris and Presta 1995). This mechanism creates a positive feedback loop involving the sequestered FGF-2 in ECM that will be freed and thus activates migration and proliferation of cells. FGF-2 is also able to induce integrin $\alpha_V\beta_3$, initiating MAPK signaling. This pathway is thought to enhance EC migration (Shono *et al.* 2001).

B.IV.2.d.β. FGF-2 and transformation

FGF-2 is considered a powerful activator of cell proliferation. This aspect of FGF-2 is applicable both to ECs and tumour cells because they express FGFRs and trigger proliferation pathway . However to note, in cancer this signal transduction is altered and exacerbated by FGFR constitutive activation or amplification, ligand independent signaling, etc.

B.IV.3. PDGF

PDGF is an important angiogenic factor involved in the biology of the mesenchymal cells such as proliferation of fibroblasts and other connective tissue cell types (Alvarez, 2006). PDGF has pleiotropic effects and has roles in embryonic development, CNS development, the vascular system, tissue homeostasis, and wound healing(Dunn, 2000). In mice, targeted disruption of PDGF-B or the PDGF receptor

ablated the microvascular pericytes that normally form part of the capillary wall(Lindahl, 1997). Due to a wide spectrum of activies – not mentioned here – overexpression of PDGF signaling can cause various diseases like liver cirrhosis, pulmonary fibrosis, atherosclerosis, etc…

B.IV.3.a. PDGF Ligands and receptors

PDGF ligands are mainly represented by with two different types of polypeptides A or B – in a family which comptises also PDGF C and D - which act in a mature form as sulfide bound dimers like PDGF-AA, -AB or –BB. Theses angiogenic factors are principally paracrine factors, but also can act as autocrine factors in tumors. To be secreted, PDGF-A and B are cleaved at their amino terminal domain. In many tymor types such as glioblastomas, PDGF-BB is overexpressed and lead to hypervascularization (Cao, 2008). PDGF expression is responsive to various simuli such as hypoxia, thrombin, cytokines, and growth factors, including PDGF itself (Andrae, 2008). Likewise FGF-2, Diffusion of PDGF in the tissue interstitium is regulated by binding to extracellular matrix components like HSPG.

PDGF ligands bind tyrosine kinase receptors, the PDGF-α and PDGF-β receptors - which chains can as well homo or heterodimerize. Both receptors can activate many of the same major signal transduction pathways, including PI3K, Ras MAPKinase and PLCγ pathways, but have a different pattern of activation depending on cell types. PI3K is essential for cell migration, actin remodeling and survival. Ras pathway on the contrary lead to cell proliferation. As VEGF and FGF-2 receptors PDGF receptors undergo dimerization and autophosphorylation to activated the subsequent transduction signal pathways. They are expressed in fibroblasts, pericytes, Vascular SMCs, platelets, neurons, mammary epithelial cells, macrophages, myoblasts and others.

B.IV.4. TGF-β

Transforming growth factor β family is constituted by 35 members in humans in which the typical member is TGF-β1. In this respect, depending on the context, TGF-β induce an extreme variety of effect ranging from stimulation and inhibition of cell growth, extracellular matrix elaboration, EC migration incduction but also maturation of mural cells and regulation of immune. TGF-β signaling is of first importance in cell

biology and angiogenesis a it is responsible for many diseases such as cancers, pulmonary and liver fibrosis and vascular disorders (Bertolino, 2005).

B.IV.4.a. TGF-β pathways

TGF-β members exert their effect by binding to couples of specific serine/threonine kinase type I – also termed activin receptor-like kinase – ALK - and type II receptor complexes. TGF-β has high affinity for the TGF-β type II receptor - TβRII - and on binding a specific TGF-β type I receptor - TβRI - is recruited and phosphorylated by TβRII. This reaction triggers the the TGF-β signal transduction mediated by *smads* proteins – R-smads like Smad 2/3, Smad 1/5/8 and Co-smad like smad4 – which translocate to the nuleus and with co-factors activate gene transcription.

TGF-β is of first importance in angiogenesis as studies have shown that loss TGF-β signaling induce abnormal differenciation and maturation of the primitive vascular plexus, resulting in fragile vessels with decreased integrity of the vessel wall (Goumans, 2003). the vessels of the TGF-β1 mutant embryos have decreased wall integrity, which is caused by a defect in endothelial cells differentiating into capillarylike tubules.

The main difficulty for the understanding of TGF-β role in angiogenesis is that it is able to have opposite effect depending on the context. In physiological conditions TGF-β is though to induce the proliferation of ECs during the activation step of angiogenesis and also participate in tube vessel formation by inhibition cell proliferation in the resolution step. This bipolar effect is reinforced by observations that low doses of TGF-β induce cell proliferation whereas high doses inhibit it (Pepper, 1993). Otherwise TFG-β is ale to induce MMP-2 and MMP-9 expression, matrix remodeling and release of proangiogenic factors by monocytes.

B.V. p53 and angiogenesis

Because of its implications in other mechanism of cell defense against aggressions and all aspects of tumour progression, assuming a direct role of p53 in angiogenesis has been more difficult to prove. In 1998 Holmgren *et al.* showed that independently of its anti-proliferative and proapoptosis role p53 is able to induce dormancy by inhibiting angiogenesis (Holmgren *et al.* 1998). However p53 plays a key role in the inhibition of angiogenesis by interacting with HIF-1α under hypoxic

conditions, inhibiting antiangiogenesis targets and activating proangiogenic ones and also acting directly upon regulation of proliferation and migration of ECs. Likewise, other tumour suppressors such as retinoblastoma and pVHL do have antiangiogenic properties, frequently inactivated in tumour cells.

B.V.1. Correlations between angiogenic behavior and p53 status.

Figure 21. p53 involvement in angiogenesis and consequences of its loss.

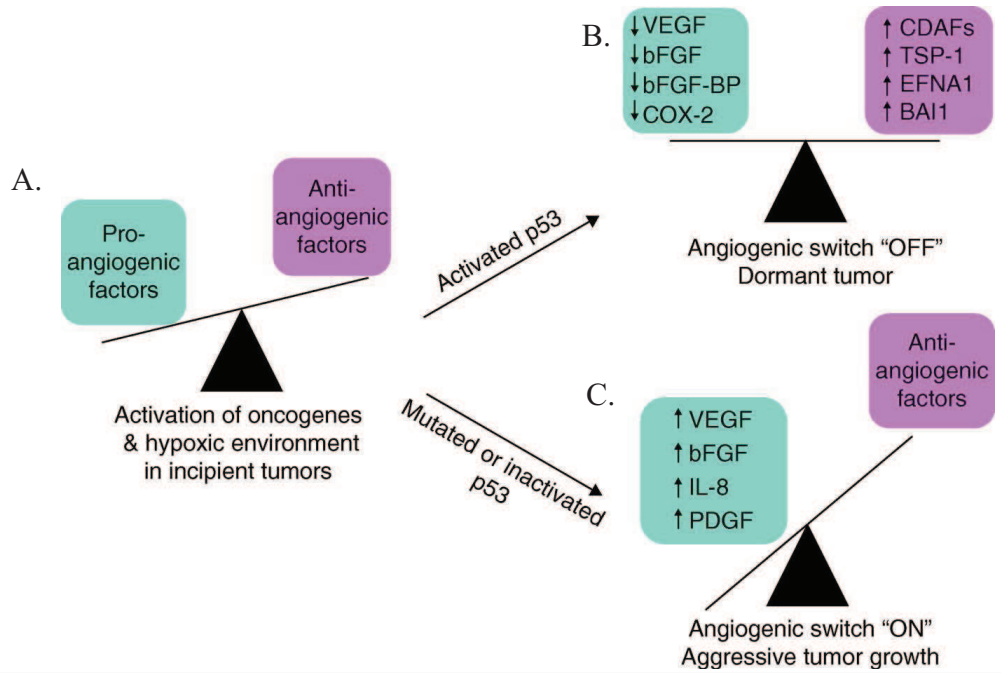


Diagram showing the innate balance of pro and antiangiogenic genes in p53 target genes in neoplasms – A, and the consequences of p53 activation – B. Target genes shown here will be discuss more in details in this report. When p53 is inactivated or mutated, tumour can escape dormancy and activate their angiogenic program – C. Shown here important proangiogenic genes.

p53 status is an important prognosis marker for tumour growth, aggressiveness and metastasis potential, due to its classical implication towards the different mechanisms of response to cellular stresses. Regarding the evaluation of tumour angiogenicity linked with p53 status, several studies have revealed strong correlation in various types of cancers such as colon cancer, head and neck cancers, breast cancers and NSCLC cancers (Linderholm *et al.* 2000; Yuan *et al.* 2002; Perrone *et al.* 2004). Two independent studies in human prostate cancers have shown that tumours expressing mutated p53 have significantly greater microvessel density – MVD – than tumours expressing wild-type p53 (Yu *et al.* 1997; Takahashi *et al.* 1998). These clinical observations have constituted the first steps towards the understanding of p53 antiangiogenesis in cancer – Figure 21.

B.V.2. p53 and hypoxia.

Under strong hypoxic conditions near anoxia – around 0.2 % of O2 – p53 response possesses a unique pattern. Contrarily to other p53 dependent stresses, p53 interferes in angiogenesis with the hypoxia inducible factor HIF-1α as explained earlier on (Ravi *et al.* 2000). This crosstalk allows us to better apprehend the mechanisms underlying the different responses to hypoxia. This important balance bears some common targets genes regulated in an opposite fashion such as VEGF, GLUT-1 and Bcl-2 that would explain the radical changes in response from cell survival to cell death (Ebert *et al.* 1995; Forsythe *et al.* 1996; Zhang *et al.* 2000; Wu *et al.* 2001; Schwartzenberg-Bar-Yoseph *et al.* 2004; Zhang and Hill 2004). In the same time a specific pattern in p53 response encountered in hypoxia may be explained by NO levels, and phosphorylation in p53 S15, as explained earlier on. Additionally, some groups have demonstrated that hypoxic stress does not induce both p21 nor MDM2 target genes and fails to induce the acetylation of Lys382 of p53 which is known to be important for p53-p300/CBP interaction (Koumenis *et al.* 2001; Hammond *et al.* 2006). However hypoxic activation of p53 results in increase of mSin3A affinity, a p53 transcriptional corepressor (Murphy *et al.* 1999). Another factor involved is the BNIP3L, a p53 target gene induced as well in hypoxia (Fei *et al.* 2004). Yamakuchi *et al.* identified a miRNA – miR-107 – regulated by p53, which inhibits hypoxia signaling by suppressing HIF-1β expression (Yamakuchi *et al.* 2010). They showed a miR-107 induction by p53 with etoposide treatment. Interestingly loss of ATM induces the upregulation of HIF-1α through oxidative stress (Ousset, 2010 #964). Concomitantly with p53 activation, ATM by this mechanism controls tightly two major factors in angiogenesis and tumour suppression.

In conclusion, mild hypoxia result in stabilization of HIF-1α but not p53, allowing the cell to trigger survival and a proangiogenic response. But when the conditions of hypoxia are too severe, p53 is also stabilized through formation of a complex containing HIF-1α and other factors like MDM2, pVHL or p300 and p53 S15 phosphorylation by NO. p53 is activated but does not induce cycle arrest and rather an inhibition of its target genes. However lack of evidence in these conditions remains to define p53 target genes involved in hypoxia-induced apoptosis. On must note that Hammond and Giaccia localized p53 in the cytoplasm after induction by the hypoxia-mimetic deferroxamine (Hammond and Giaccia 2005). The remaining uncertainties rely

also on the definition of hypoxic and anoxic conditions which may vary between studies. For instance, Kamat *et al.* compared acute and chronic hypoxia – 4 and 72 hours – and showed that the apoptotic response triggered in the cells in chronic hypoxia was caused by p53 (Kamat *et al.* 2007).

B.V.3. Regulation of proangiogenic targets.

One of the principal target of p53 towards proangiogenic factors is VEGF-A. Indeed this latter is a p53 target gene through competitive binding of the transcriptional VEGF-A co-activator SP1 (Zhang *et al.* 2000; Pal *et al.* 2001).

Similarly p53 is able to inhibit the FGF-2 transcription through binding to its basal promoter (Ueba *et al.* 1994). Our lab has shown that p53 is also able to bind the 5'UTR IRES region of the FGF-2 mRNA and to inhibit its translation by a modification of the mRNA secondary conformation (Galy *et al.* 2001; Galy *et al.* 2001). Adding a layer to p53 control upon FGF-2 biosynthesis and metabolism, p53 downregulates the expression of the FGF binding protein – FGF-BP (Sherif *et al.* 2001). This protein is involved in the activation of FGF-2 particularly when bound to HSPG in the ECM. Finally p53 is able to repress heparanase, a cleaving enzyme which frees VEGF-A and FGF-2 (Baraz *et al.* 2006 626).

p53 downmodulates COX-2 expression by competing DNA binding with TATA binding protein (Subbaramaiah *et al.* 1999). COX-2 is an inducible enzyme that converts arachadonic acid to prostaglandin H_2, a progenitor of prostanoids, which are key players in inflammation and actually also promote the expression of proangiogenic factors (Subbaramaiah *et al.* 1999; Iniguez *et al.* 2003).

Cysteine-rich 61 – Cyr61 – and p53 status have been strongly and negatively correlated in prostate cancer (Lv *et al.* 2009). Cyr61 is a member of the CCN protein family that has been implicated in diverse biological processes, promoting for example cell adhesion and proliferation, angiogenesis, and tumourigenesis. In this study authors also showed that overexpression of wildtype and mutant p53 in various cancer cell lines confirmed that p53 tightly regulate Cyr61 expression.

B.V.4. Regulation of antiangiogenic targets.

The involvement of p53 in the angiogenic switch non solely implies inhibition of proangiogenic genes but also enhancement of antiangiogenic ones. Interestingly, antiangiogenic genes have double actions through the ECs. First, they inhibit EC

proliferation, migration and rearrangement but also induce EC apoptosis. One would argue that this induction by p53 is merely differentiable to the classical p53 apoptosis pathway. Interestingly p53 antiangiogenic target genes are almost presents in the ECM – Table 2.

Thrombospondin-1 – TSP-1 – is the first antiangiogenic factor identified as a p53 target gene and one of the first p53 target genes (Dameron *et al.* 1994). TSP-1 is a 450 kDa glycoprotein, in the ECM, which inhibit angiogenesis by ECs inhibition of proliferation and migration *via* its receptor CD36 (Ren *et al.* 2006). Dameron *et al.* showed that its expression is strongly correlated with the dormancy state of the tumour and thus is an essential factor regulated by p53. This work has been confirmed by two groups showing similar effects of p53 on TSP-1 in a p53 null context (Giuriato *et al.* 2006; Su *et al.* 2010).

Table 2. p53 target genes in angiogenesis	**Reference**
p53-downregulated genes	
VEGF-A	(Pal *et al.* 2001)
bFGF	(Ueba *et al.* 1994)
bFGF-BP	(Sherif *et al.* 2001)
Cyr61	(Lv *et al.* 2009)
COX-2	(Subbaramaiah *et al.* 1999)
p53-upregulated genes	
TSP-1	(Dameron *et al.* 1994)
BAI1	(Nishimori *et al.* 1997)
EPHA2	(Dohn *et al.* 2001)
ephrin-A1 – EFNA1	(Dohn *et al.* 2001)
α1 collagen 18 – COL18A1	(Miled *et al.* 2005)
α1 collagen 4 – COL4A1	(Wei *et al.* 2006)
SEMA-3F	(Futamura *et al.* 2007)
P4HA2 [α(II) 4-prolyl hydroxylase]	(Teodoro *et al.* 2006)

Semaphorin-3F – SEMA-3F – when overexpressed inhibits cell growth and tumour related angiogenesis. Futamura *et al.* identified it as a direct p53 target gene and showed strong correlation between p53 status, SEMA-3F and its receptor neuropilin 2 – NRP2 – levels (Futamura *et al.* 2007). Overexpression of SEMA-3F leaded to inhibition of tumour growth and decrease of MVD levels in colon cancer xenografts.

Brain-specific angiogenesis inhibitor-1 – BAI-1 – is a large transmembrane protein of the B family of G-protein coupled receptors which was originally identified

in a screen for p53-induced genes in glioblastoma cells (Van Meir *et al.* 1994; Nishimori *et al.* 1997; Nishizaki *et al.* 1999). The first group to identify BAI-1 was Van Meir in 1994, who found an antiangiogenic factor secreted in condition mediums, following overexpression of p53. Still, they were not able to characterize it. BAI-1 contains TSP-1 repeats – TSR – and is processed into an antiangiogenic fragment called vasculostatin present in the ECM – and responsible for the effects observed by Van Meir (Kaur *et al.* 2005).

Ephrin receptor A2 – EPHA2 – and its ligand ephrin A1 – EFNA1 – are also transcriptionally induced by p53 (Brantley *et al.* 2002; Kaur *et al.* 2005). EPHA2 is a part of the tyrosine kinase receptor family ephrin receptors and plays important roles in angiogenesis, but also in cancer progression. EPHA2 signaling is a complex mechanism where the preservation of the ligand-receptor ratio is determinant for antiangiogenic induction. p53, promoting concomitantly EPHA2 and EFNA1, uses the ephrin pathway to induce antiangiogenesis.

Finally miR-34a, a p53 target miRNA, has been shown to induce senescence of EPC (Zhao *et al.* 2010). This would give another important role in angiogenesis besides its effects on cell cycle arrest, apoptosis and senescence.

B.V.5. Activation of collagen derived antiangiogenic factors.

Collagens, principal component of the ECM, greatly contribute to the vessel remodeling during the basal membrane breakdown. In their mature form i.e. a triple helix composed of members amongst a family of 29 ones, cross-linked and assembled together, collagens are generally though to be scaffolds for the surrounding vessels – Figure 22. Simple chains of collagens are proline hydroxylated by α(I) and α(II) prolyl 4-hydroxylases – α(I)PH and α(II)PH – enabling hydroxide bound between filaments and strongly enhancing stability of the helix. Their overexpression induces EC stability, adhesion and migration (Form *et al.* 1986). Under certain circumstances, they are processed by different proteases to deliver antiangiogenic fragments present in the non collagenous C-terminal domain of these chains.

During collagen biosynthesis and metabolism, p53 acts directly and indirectly upon several steps, from the transcription upregulation of collagen members to the cleavage of the C-terminal antiangiogenic fragments.

Among the large family of collagen genes and isoforms, p53 is able to transcriptionally activate COL4A1, COL4A3 and COL18A1 (Miled *et al.* 2005; Teodoro *et al.* 2006; Wei *et al.* 2006). COL18A1 following cleavage by MMP-9, elastase or cathepsin L frees endostatin, a well-known antiangiogenic fragment (Wen *et al.* 1999; Felbor *et al.* 2000).

Figure 22. *Unravelling collagen.* Julian Voss-Andreae's sculpture.

p53 contributes to the formation of the collagen triple helix by enhancing the hydroxylation of proline residues – 9% of total residues – of collagens chains. It enhances the transcription of one rate-limiting enzyme, α(II)PH (Teodoro *et al.* 2006). Surprisingly overexpression of this enzyme did not lead to an increase in the amount of stable triple helix chains in the ECM but rather a diminution caused by a strong activity in collagen C-terminal cleavage. Consequently Teodoro *et al.* found an increase of tumbstatin and endostatin fragments. This study limited its investigations to these two collagen chains although their involvement in angiogenesis is predominant. The protease enzymes involved in this activity remain to be elucidated, but authors hypothesize the involvement of MMPs such as MMP-2, a p53 target gene (Bian and Sun 1997). This work may open the gate of some important findings regarding the antiangiogenic potential of p53, through collagen derived antiangiogenic fragment stimulation.

If p53 has been shown to be an essential antiangiogenic factor, growing evidence tends to explain that mutant p53 not only passively fails to be active, but takes part in complexes that induce angiogenesis. For instance formation of a complex containing mutant p53 and ESAF1 shows transcriptional activity towards the inhibitor of DNA Binding 4 – ID4 – which consequently increases IL8 and GRO-α proangiogenic factors (Fontemaggi *et al.* 2009).

B.VI. Models of angiogenesis.

Since many years angiogenesis assays are considered of prime importance for understanding and predicting the effects of specific molecules on angiogenesis.

Consequently a considerable list of assays is nowadays available for studying the different aspects of angiogenesis.

B.VI.1. In vitro models

There are several advantages of *in vitro* studies: They are highly reproducible, quick, easy to perform and in some extend, cheap. However some would argue that they are oversimplified, as they do not consider the high complexity and heterogeneity of ECs – ECs from fenestrated, sinusoid or BBB capillaries, from capillaries or arteries, from venous or arteries or from various regions of the body. Moreover, despite the existence of co-cultured ECs with pericytes, *in vitro* studies can not reproduce the whole blood vessel and stroma, including all the cell types it normally has. *In vitro* models are proliferation assays, migration assays, tubulogenesis and co-cultures.

Endothelial cell proliferation assays are common in investigation. However some care for EC handling is important and precise settings must be respected for proper experiments i.e. cell density, number of passage and induction of a quiescent state. Proliferation assessment can be achieved by cell counting, MTT – [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide], cleaved by a mitochondrial deshydrogenase enzyme to produce purple/blue formazan crystals – and DNA synthesis by measuring the incorporation of [^{3}H]Thymidine or bromodeoxyuridine – BrdU (Denizot and Lang 1986; Gomez and Reich 2003; Yu *et al.* 2004). Cell cycle analysis assays can also be undertaken in this purpose.

Migration assays involve mainly the Boyden chamber assay and the wound healing assay. The Boyden chamber is highly sensitive to angiogenic agents and this experiment has the advantage not to last long time – few hours – but setting up the Boyden chamber requires some attention. On the contrary, the wound healing assay does not require the mounting of a chamber and two compartments but just needs a scraping into the Petri dish containing ECs. However, “wound repair” in this model is a multi-step process involving spreading, proliferation and migration events, and it has to be considered that the scraping constitutes a stress for the cells (Wong and Gotlieb 1988).

Endothelial cell differentiation constitutes the late step of angiogenesis and is represented by capillary like tubule formation. This assay involves plating a layer of ECs onto ECM like matrix – pig matrigel, fibronectin, etc. Compounds or conditioned

media are then added to the cells and tubule formation is assessed within few hours. This assay is widely used for measuring the potency of anti- or proangiogenic molecule.

Co-culture assays are mainly used for studying the interactions between ECs and mural cells. In summary, ECs are first coated then usually pericytes are added and several measurements can be done such as proliferation, and cell to cell interactions.

B.IV.2. Ex vivo models

Contrary to *in vitro*, *ex vivo* models allow inclusion of an entire organ in the experiment. Generally all cell types are represented as well as the surrounding stroma and *in vivo* angiogenesis is observed.

Discs – like aortic, vena cava and lymphatic rings – or sections of the specific tissue type are cultured in a matrix and are monitored for microvessel outgrowths for two weeks. Number and length of outgrowths, the area covered by outgrowths and the number of branches are used for quantification of angiogenesis – or lymphangiogenesis (Nicosia *et al.* 1997). In these models ECs are not altered by successive passages and are quiescent. However one discrepancy is the absence of different factors – and in the case of vascular rings the absence of circulating cells. A similar assay is the chick aortic arch model which is collected from embryonic aortic arch at D17.

B.VI.3. In vivo models

- The corneal angiogenesis assay. The cornea is a non vascularized and transparent tissue easily available for experiment. These properties make it a model of choice for angiogenesis *in vivo* assays as angiogenesis is easily visible and quantifiable (Gimbrone *et al.* 1974). As almost all types of corneal injury induce neovascularization, implantation of micropocket intracorneally next to the limbal epithelium containing substances, tumour cells or extracts in order to evaluate their angiogenic potential, or to test systemic injection or antiangiogenic compounds. Vascularization is visualized and quantification by adding fluorescent dye or India ink in flattened corneas onto slides. In this model, angiogenesis is quite atypical, as cornea is normally avascular and does not sense oxygen. This model raises however some ethical concerns.
- Sponges and polymers. Many materials can be implanted into animals such as stainless steel mesh chambers, hollow chambers with porous walls, synthetic sponge matrix – polyvinyl alcohol, polyester and polyurethane – implants or Matrigel plugs. The matrigel – liquid at 4 °C and solid at 37 °C – is composed of different

proteins of ECM and the basal membrane, plus growth factors, although now these matrigels are reduced in growth factors. Compounds or cells can be suspended with it prior to subcutaneous injection into mice. Angiogenesis is measured by CD-31 positive vessel by IHC, hemoglobin content, FITC-dextran injections in mice, etc.

- The dorsal air sac model. First proposed by Oikawa in 1997, this model consists in a chamber ring loaded with tumour cells on the murine skin – in the dorsal part (Oikawa *et al.* 1997). Quantification of angiogenesis is possible in five days by injection of Evan blue into the mice, staining only pre-existing vessels. Blood volume can be measured by circulating Cr51-labelled erythrocytes.

B.VI.4. The chick embryo chorio-allantoic membrane – CAM

B.VI.4.a. Function and structure in embryonic development.

The CAM is a transitory extra-embryonic organ resulting from fusion of the chorion and the allantoidic layers at day 4 post fertilization and contains an extremely dense capillary network devoted to supply the embryo of oxygen, calcium from the shell and other nutrients, and carries carbon dioxide excretion. From day 5 and until hatching – day 21 – the CAM undergoes several steps of angiogenic development and reduction: During three days the angiogenic network grows very rapidly by sprouting with immature BVs lacking basal lamina and SMCs, setting up the first capillary plexus. From then the capillary network attaches to the basal membrane of the epithelial layer of the ectoderm part of the CAM adjacent to the shell membrane. It continues to expand its capillary network until day 12 by intussusceptive angiogenesis. Thereafter, EC proliferation quickly declines, network architecture is mature but will adopt its final arrangement on day 18 (Ausprunk *et al.* 1974). Javerzat *et al.* have investigated the molecular regulations during CAM development and found specific patterns of expression correlating to the above mentioned steps such as BMP4 and FGFR2 upregulation from days 7 to 10 (Javerzat *et al.* 2009)

A dense lymphatic network also exists on the CAM. This allows the draining of metabolites but it may not have the same functions as in human adults because immune system is not mature. Indeed it enables the implantation of biopsies, xenografts of tumour cells, molecules and different sterile objects onto the CAM without conferring immune response.

B.VI.4.b. The CAM as a tool.

Table 3. Tumours inducing angiogenesis in CAM model

Tumour	**Authors**
Adenocarcinoma of the endometrium	(Palczak and Splawinski 1989)
B-cell non-Hodgkin's lymphoma	(Ribatti *et al.* 1990)
Glioblastoma	(Klagsbrun *et al.* 1976)
Head and neck squamous cell carcinoma	(Petruzzelli *et al.* 1993)
Hepatocellular carcinoma	(Marzullo *et al.* 1998)
Lipoma	(Lucarelli *et al.* 1999)
Melanoma	(Auerbach *et al.* 1976)
Meningioma	(Klagsbrun *et al.* 1976)
Neuroblastoma	(Ribatti *et al.* 2002)
Walker 256 carcinoma	(Klagsbrun *et al.* 1976)
Tumour Cell type	**Authors**
B- Melanoma cells	(Takigawa *et al.* 1990)
Ecs isolated from patients with multiple myeloma	(Vacca *et al.* 2003)
Lymphoblastoid cells	(Vacca *et al.* 1998)
Mammary tumour cells transfected with VEGF	(Ribatti *et al.* 2001)
Neuroblastoma	(Ribatti *et al.* 2002)
Plasma cells from patients with multiple myeloma	(Ribatti *et al.* 2003)
Walker carcinoma 256 cells	(Klagsbrun *et al.* 1976)

Tumours and tumour cell types successfully implanted in Chicken CAM. From (Ribatti 2008)

The CAM has been developed since more than one century and has greatly proven its usefulness for the studies of angiogenic compounds and tumour growth, dissemination and metastasis – Table 3. Generally studying the effects of a molecule on angiogenesis takes only two or three days whereas the effects of tumour cell can be studied over two to seven days. Compared to other *in vivo* assays this gain of time is valuable. Observations and experimental manipulations at different times are easily manageable. Several types of tumour and tumour cell types but also non tumoural cells and compounds have been successfully implanted onto CAM – Table 3 (Ribatti 2008).

CAM development can be monitored through *in ovo* or *ex ovo* follow-up. This latter consists in discarding the shell to observe the embryo development in a Petri dish. It confers the advantage of a larger window of observation for the CAM and also the possibility of multiple grafting onto the CAM, but this step lowers the proportion of

viable and alive embryos throughout the experiment. The quantification of angiogenesis can be performed by several counting means, such as number of vessels, number of branches, scoring the angiogenic net changes, microvessel density, uptake of H3 Thymidine, etc. However angiogenesis must be segregated from vasodilatation which is not in all counting methods, possible. SNA-lectin or LCA-lectin for ECs staining, desmin for pericytes, α-SMA for SMCs; all these cell markers are available for CAM immunohistochemistry and immunofluorescence.

Depending on the cell type, CAM can be used for studying the dissemination of tumour cells into internal organs of the embryo such as lung, liver and brain. Indeed within five to seven days aggressive tumour cells develop to sizable tumours, escape the primary site, invade surrounding stroma, intravasate into BVs and reach distal part of the and embryo organs, summering spontaneous metastasis in a very short period of time. Fluorescent tagged cells can be followed for distal dissemination as well as quantification of *Alu* DNA repeats, specific of primate genome (Deryugina and Quigley 2008).

C. Glioblastomas

C.I. Introduction to glioblastomas

Primary brain tumours from the central and peripherical nervous systems – CNS and PNS – are derived from cells of neuro-ectodermic origin and constitute, with brain tumour metastases, a relatively rare tumour type – 2% of all types of tumours. Their recognition and characterization are funded upon morphologic and immunohistologic bases (Dracopoli *et al.* 1985; Kleihues *et al.* 2002). There are twelve main categories of brain tumours and up to one hundred sub-categories. The most frequent tumours in the CNS are grouped into the generic term gliomas but are astrocytomas, oligodendrogliomas, oligoastrocytomas or mixed gliomas. As showed in Table 4 gliomas can correspond to very different types of tumours. This term is often confused with the most prevalent type of brain tumours glioblastomas or glioblastoma multiform – GBM – which represent 40% of primary malignant brain tumours.

Table 3. Different types of brain tumours

Tumours	Type of origin	Tumour grade – from WHO
Gliomas	Astrocyte	Anaplasic astrocytoma GII diffuse astrocytoma GIII Anaplasic astrocytomas GIV Glioblastoma
Oligodendro-gliomas	Oligodendrocyte	GII Oligodendroglioma GIII Anaplasic Oligodendroglioma
Mixed gliomas	Astrocyte or oligodendrocyte	GII oligo-astrocytoma GIII Anaplasic oligo-astrocytoma
Ependynomas	Ependymocyte	GII Ependynoma GIII Ependynoma
Embryonic tumours		GIV Neuroblastoma GIV Meduloblastoma

WHO classification of brain tumours. From (Kleihues and Sobin, 2000)

Unfortunately, GBM and other primary tumours are going increasingly these last years (Brandes *et al.* 2003). GBM is of bad prognosis with a 5% survival rate after five years.

On a histopathologic view, glioblastomas are highly cellular, anaplastic, with undifferentiated, round, atypic and pleiomorph astrocytes. GBM are highly proliferative and characterized by important vascular glomeruloid proliferations and/or

pseudopalisading shaped necrosis with marginal tumour cells in the surrounding forming a palisade. Ki-67 index is generally superior to 20%. Various symptoms are caused by GBM: head aches, nauseas, vomiting which traduce intracranial hypertension, focal neurological defaults due tumour infiltration and troubles in the behavior (Grossman and Batara). These tumours are extremely aggressive and invasive but they do not disseminate systemically and death is the consequence of local expansion.

Various favorable prognostic factors include age, KPS – Karnofsly performance status – histology, absence of extended necrosis, and the size of the residual tumour following chirurgical resection – debulking (DeAngelis). However GBM is still extremely deadly (Grossman and Batara).

There are two sub-types of GBM: primary GBM and secondary GBM whether the genetic and clinical characteristics. Primary GBM develops extremely rapidly – in six months – from glial cells and is the spreadest in elder patients. The secondary GBM is developed in months or years after diagnosis of low grade astrocytomas and affects mainly younger people. Primary GBM oncogenesis is a genetic multistep mechanism such as EGF amplification, loss of chromosome 10, amplification of MDM2 gene and deletion and mutation of PTEN gene (Ohgaki 2005). On the contrary secondary GBM oncogenesis involves p53 inactivation and overexpression of EGF and PDGF pathways. Due to their high tumour grade and their capacity to become resistant from classical methods, targeting the microenvironmental cells and in particular ECs that are more genetically stable is a well suited strategy.

C.II. Angiogenesis in glioblastomas.

One important and unique pattern in the brain vascular organization is the presence of a merely impermeable barrier between blood and stoma called brain-blood barrier – BBB. This barrier is constituted of ECs, pericytes and astrocytes and constitutes a strong obstacle to drug delivery. In brain tumours this barrier loses its integrity allowing tumours cells to induce neoangiogenesis. Disruption of BBB, increase of tumour vessel permeability and accumulation of fluids in the surrounding brain induce vasogenic edema, the major cause of morbidity. In the case of gliomas, vascularization is strongly correlated with tumour grade and prognosis (Leon *et al.* 1996). Consequently, antiangiogenic treatment in brain tumour hampers non-solely

tumour development but also the above mentioned related complications of BBB loosening. Hispathologically, GBMs are characterized by microvascular proliferation of 12.5 % of total ECs, which is 40 fold the proliferation index of normal vessels. Newly formed tumour ECs have thicker cell wall due to hyperplasia, vascular permeability, abnormal pericyte layer distribution and thinner basal membrane.

If glioblastomas can display strong vascular networks, they still have hypoxic regions resulting from the hyperproliferation of these tumour cells and microthrombosis phenomenons. The low levels in oxygen is responsible for the VEGF overexpression and stabilisation of HIF-1α at these juxta-necrotic regions, in the palisading glioblastomas cells (Plate *et al.*). Constantly proangiogenic factors such as EGF, TGFα and HGF induce VEGF expression *in vitro* (Goldman *et al.* 1993; Moriyama *et al.* 1998; Valter *et al.* 1999). Loss of PTEN, p53 and pVHL and activation of the oncogenes ras and src induce also VEGF expression (Jain *et al.* 2007).

Interest in anti-VEGF therapies has been rekindled by preclinical data showing that, when used judiciously, these agents can transiently 'normalize' the tumour vasculature and potentially reduce the risk of haemorrhage, enhance the penetration of concurrently administered chemotherapeutics and improve the efficacy of cytotoxic drugs. Increased oxygenation could also reduce the invasive potential of cancer cells.

C.III. Treatment of glioblastomas.

Glioblastoma is one of the most lethal tumours, despite intensive research for many years. The proportion of patient curing from it is very low, under 10%. However research has permitted to considerably lengthen overall survival. Common treatments of glioblastomas involve repetitive surgery, local irradiations and systemic chemotherapies.

Surgical excision is still the most prevalent treatment for brain tumours. However the beneficial aspects of local surgery have somehow raised some doubts concerning their efficacy to lengthen survival. Some of the reasons are the extreme difficulty to entirely resect GBM because of their extensive three dimensional structures and sometimes surrounding benign tumours can be activated by surgery. For these reasons surgery is followed by radiotherapy or chemotherapy to avoid recurrence.

Radiotherapy is considered as a basic treatment for selected gliomas. Particularly gamma rays are well suited for profound tissues and adapted for brain tumours. Fourty

five to sixty Gy radiotherapy treatment for six week can enhance median survival from twenty weeks to one year.

Chemotherapy is also a good alternative as it ameliorates survival and saves the functionality of the surrounding replacing totally or partially the local treatment. The BBB only allows small molecules like alkylant agents and platinum derivates. Irradiations with alkylant agent temozolomide orally have become one of the standard treatments for newly diagnosed GBM. This latter have little impact on outcome as median survival is 2.5 months more than surgery and radiotherapy without chemotherapy which is of 12.1 months (Stupp *et al.* 2005).

C.III.1. Antiangiogenic therapy

The main issues concerning radio and chemotherapies in glioblastomas come from the ability of these cells to overcome aggressions that are lethal in many tumours types, the heterogeneity of cells within a tumour and between patients, GBM localization underneath the BBB and into the brain, render difficult to obtain full efficiency of these treatments. Likewise, although some consensus have risen in the conditions of radio and chemotherapy treatment, defining the best conditions that give maximum tumour cell death and minimum secondary effects depending on the different GBM sets is still an ongoing task. Contrarily to it, antiangiogenic therapy targets ECs and pericytes from the surrounding of the tumour, cells that are more genetically stable and are less prone to develop a resistant phenotype to treatment. Another important aspect of antiangiogenic therapy has been recently revealed. In hyperangiogenic tumour environment, BVs are only partially functional. The decrease of angiogenic potential by antiangiogenic agents induces renormalization of the vessel network and restoration of the blood flow, if the treatment is not too strong. This normalization regained helps other cytotoxic agents to reach properly the tumour and thus give full potential of treatment (Kim and Lee 2009).

As we have seen it earlier on in this report, VEGF plays a principal role in angiogenesis and tumour related angiogenesis. Thus many approaches intend to disrupt VEGF or down and upstream actors. Bevacizumab, a humanized mouse antibody targeting VEGF-A in combination with a chemotherapeutic agent is already in use for colon, lung and breast cancer. Sorafenib, vatalanib/PTK787 and Sunitinib are VEGFR tyrosine kinase activity inhibitors in RCC. Soluble receptors trapping VEGF – VEGF Trap – show also interesting properties. In brain tumour these compounds are still in

clinical trial phases. Phase II trials showed improvement of apparent median progression-free survival but phase III trials are still ongoing. Bevacizumab received accelerated FDA approval in May 2009 for treatment of high grade astrocytomas. For better understanding in extend the different open or recent clinical trial – refer to table X (Vredenburgh, 2007 #442;Winkler, 2004 #446;Batchelor, 2007 #448;Kim, 2009 #972). Both Vredenburgh *et al.* and Batchelor *et al.* showed better therapeutic results with antiangiogenic agents in combination with chemotherapeutic ones. These results are supported by several preclinical models of primary and secondary brain tumours (Yuan *et al.* 1996; Jain *et al.* 1998; Tong *et al.* 2004). However the normalization induced by the antiangiogenic agents seems to be only transient and finally reverses, providing a limited time window for chemotoxic agent action. Another hypothesis is that renormalization of blood vessel blow and structure first allow increasing the efficacy of chemotherapeutic penetration, but then brain tumours BVs come back to a more physiological phenotype i.e. tightening of BBB, the chemotherapeutic agents no longer reach the tumour spot.

Table X. Ongoing clinical Trials with antiangiogenic agents targeting brain tumours. Jan 2009

Drug	Target	Phase	Tumours
XL 184 (TKI)	VEGFR, MET	II	GBM
ZK222584 (TKI)	VEGFR, PDGFR	I,II	MG
Pazopanib (TKI)	VEGFR, PDGFR/c-	II	Glioma, Meta
Sorafenib	VEGFR, PDGFR/Raf	I,II	GBM, Meta, GS
Sunitinib (TKI)	VEGFR, PDGFR	I,II	GBM, Meta
Thalidomide/Lenalidom	VEGF/bFGF/TNF	I,II	GBM, Meta
VEGF Trap (Peptide)	VEGF	II	MG
Ceradinib (TKI)	VEGFR	I,II,III	GBM
ZD6474 (TKI)	VEGFR	I,II	GBM
CT-322 (Peptide)	VEGFR	I,II	GBM
Bevacizumab (mAb)	VEGF	I,II	GBM
Valproic Acid	Potential	II	Meningioma, GBM
Everolimus/Sirolimus	FKBP-12/mTOR	I,II II	MG GBM
Cilengitude (Peptide)	Integrin	II,III	GBM, MG
RAD001 (SKI)	mTOR	III	Astrocytoma
Temsirolimus (SKI)	mTOR	I	GBM
Tandutinib (TKI)	PDGFR	I,II	GB, Astrocytoma,
XL765 (SKI)	PI3K/mTOR	I	Oligodendroglioma
Enzastaurin (SKI)	PKC	I,II	GBM
MPC-6827	Microtubule	I,II	GBM
CY997	Tubulin (microvessel)	I,II	GBM, Gliosarc, Meta
Sodiumthiosulfate/Man	BBB	I,II	OG, OA, MB, NB,

From (Kim and Lee 2009). GS: gliosarcoma; MG: Malignant Glioma; OG: Oligoglyoma; OA: Oligoastrocytoma; MB Medulloblastoma; NB: Neuroblastoma; Meta: Metastasized secondary brain tumors; mAb: monoclonal antibody; PDGFR; Platelet derived growth factor recpeptor; SKI: Serine-Threonine kinase Inhibitor; FKBP-12: FK506 binding protein 1A-12 kDa.

Association of radiotherapy with antiangiogenic treatment looks also promising for clinical trials as some interesting findings show that VEGF inhibition enhanced the cytotoxic effect of radiotherapy and also induce ECs apoptosis (Gorski *et al.* 1999; Garcia-Barros *et al.* 2003). Interestingly radiotherapy induces HIF-1α and thus its downstream target VEGF, essential for ECs survival.

Other angiogenic pathways have been investigated in clinical trials: Inhibitors of PKC and PKCβ – Tamoxifen, Enzastaurin - Integrins antagonists – Cilengitide – HIF-1a inhibitors – Panzem – and Cyclooxygenase 2 inhibitors – Celecoxib (Reardon *et al.* 2005; Reardon *et al.* 2005; Penas-Prado and Gilbert 2007).

C.III.2. Current limitations of antiangiogenic therapy in glioblastomas

Despite the complementarity of antiangiogenic and chemoactive treatments antiangiogenic therapeutic agents have one major disadvantage for the treatment of GBMs. These agents are only able to induce a cytostatic state within the tumour, meaning it induces tumour cells to stop their growth and enter dormancy. They are not suitable for the suppression of malignant tumours. This also means that for antiangiogenic treatments patients will be always clinically followed since their treatment. Patients with cardiovascular and cerebrovascular ischemia could undergo severe secondary effects (Folkman 2006). Another aspect of importance, related to all therapies targeting a specific molecule, is the activation of parallel pathways inducing angiogenesis after the blockade of a selected one. To overcome this matter, the use of drugs that have several targets, or the use of a combination of different antiangiogenic drugs could permit to optimize the efficiency.

Another issue is the correct evaluation of antiangiogenic treatment with current means of GBM observation. Gadolinium-DTPA – Gd-DTPA – uptake observed by MRI in brain tumours is strongly affected by antiangiogenic agents. However this does not correctly fit with tumour regression. To palliate the imperfection of these technologies, new imaging modalities have been developed but need to be confirmed (Verhoeff *et al.* 2009). Antiangiogenic treatment induces instead of a shift from highly proliferative tumours to necrosis and apoptosis, a shift to invasive and angiogenesis-independent phenotype.

Viable and proliferative GBM cells are often encountered in the surrounding of a blood vessel, and also within the vessel ring. As shown previously GBM cells are able to do co-option in order to increase metabolic income. The newly formed co-opted

vessels are not sensitive to antiangiogenic treatment (Leenders *et al.* 2004; Martens *et al.* 2008). GBM can still migrate and invade proximal or distal part of the cerebrum even during antiangiogenic treatment. These clinical observations are confirmed by preclinical studies in GBM tumours for example (Du *et al.* 2008; Ebos *et al.* 2009; Paez-Ribes *et al.* 2009).

Finally some strong differences are observed concerning the measurement of brain blood flow by MRI settings in humans, i.e. significant decrease of blood flow, and the normalization hypothesis of brain blood flow which normally would lead to an increase of selected intracranial regions where antiangiogenic agents counteract angiogenic stimuli by brain tumours.

C.IV. Glioblastomas and experimental models.

C.IV.1. Mouse models

In vivo models for gliomagenesis consist mainly in tumour cells xenografts injected subcutaneously or orthotopically in immunodefiscient mice. In my following experiments we chose the U87 cells because it is a predominant cell model for glioblastoma, it is also well characterized in the CAM models and it is wild type p53. These cells come from GBM of a 44 years old women with less chromosomic aberrations than other glioblastoma cell lines (Mulholland *et al.* 2006). Spontaneous glial tumours can be obtained in genetically modified mice overexpression *src* gene in astrocytes, or mice heterozygous for p53 and NF1. However these models presents several counterparts such as time of experiment, lack of appropriate mean for measuring all the aspects of angiogenic and size evolution and differences between experimental GBMs and human GBMs.

C.IV.2. CAM model of glioblastoma.

Pretreatment of GBM cells by doxorubicin inhibited U87 MG tumour formation in a CAM assay up to 50% (Stan *et al.* 1999). Similarly amyloid beta peptide avoided tumour angiogenesis in this *in vivo* model (Paris *et al.* 2004). The INSERM team U920 uses U87 MG cell line in CAM as predilection model from brain tumour experiments. They demonstrated strong similarities between experimental gliomas tumour growth in U87cells onto CAM and normal features of GBMs in humans (Hagedorn *et al.* 2005). They also put into CAM human biopsies for further tumour characterizations. They

showed that combined IL-6 and VEGF-A inhibition by SiRNA inhibited tumour growth, angiogenesis and invasiveness, whereas one these factors solely inhibited induce tumours regression but increase of invasiveness (Saidi *et al.* 2009). Inhibition of VEGFA by siRNA was further investigated and correlation with markers of poor survival and prognosis such as CHI3L1 and PI3/elafin was demonstrated (Lemiere *et al.* 2008).

In consequence, this model bears many advantages like established and know cell line for implantation, quickness, cost and feasibility, less ethical concerns compared to mice models, and use of many experimental procedures for investigation of angiogenesis and tumour progression parameters that pushed me to develop this model in my team in Toulouse.

PROBLEM

Since its discovery in 2005, the Δ133p53 isoform has revealed important aspects of its activity and its strong interaction with p53. Acting like a dominant negative towards p53, its presence reduces considerably the transcriptional activity of p53 for its target genes such as p21 and BAX (Bourdon *et al.* 2005). It has been shown that this isoform, the Δ40p53 isoforms and the zebrafish isoform Δ113p53 corresponding to Δ133p53 in human, are able to considerably modify the activity of its big brother p53 (Chen *et al.* 2009; Grover *et al.* 2009). On the other hand, p53 is implicated in many processes such as the ones explained above – cell cycle arrest, apoptosis, DNA repair and angiogenesis – and others I didn't get into details in this report – for instance autophagy (Crighton *et al.* 2006). Although the effect of p53 in angiogenesis is somehow shrunken by its role in tumour suppression and inhibition, p53 greatly participates in angiogenesis inhibition, resulting in tumour inhibition. Thanks to the respective expertises in p53 isoforms and angiogenesis from the European associated laboratory, we decided to investigate further in the role of the Δ133p53 in angiogenesis. The idea of an effect of Δ133p53 isoforms has been triggered in the first times by a collaboration with Pierre Roux's laboratory in Montpellier who showed us an increase in migration – and potently invasion – in cells overexpresssing Δ133p53 isoforms – unpublished data, and by preliminary clinical data concerning Δ133p53 isoforms correlated with the survival rate.

Further characterization of the Δ133p53 isoform was however an essential task to undertake. Chen *et al.* contributed greatly to the understanding of this latter isoform thanks to their study in zebrafish. They established that it not only binds and blocks p53 activity, but rather contributes to qualify p53 activity and induce BCL2L gene independently of p53. They showed also that Δ113p53 expression is induced dependently to p53 in the embryo development – at 24 hours post fertilization – and in DNA damaging stresses such as γ-rays, camptothecin, and roscovitine. Furthermore they found two *cis* regulatory regions, namely −1041 to −1991 bp and −1 to −239 bp crucial for the Δ113p53 expression. This regulation by p53 has a deep impact on p53 mediated apoptosis as overexpression of Δ113p53 reduces drastically apoptosis in zebrafish. We concomitantly to these studies investigated the molecular aspects underlying Δ133p53 expression in humans.

We also wanted to investigate new and innovative aspects of p53 activity. p53 is well known for its transcriptional activity controlling the cell fate but it controls the expression of several protein through translation regulation, or direct binding, leading to proteasomal degradation (Ewen *et al.* 1995; Mosner *et al.* 1995; Galy *et al.* 2001). Our laboratory showed that p53 inhibits the FGF-2 expression through a mechanism of translational initiation blockade involving binding to the 5'UTR of FGF-2 and loss of mRNA conformation (Galy *et al.* 2001; Galy *et al.* 2001). Consequently, p53 is able to regulate FGF-2 by two distinct mechanisms. Many questions have risen from these observations: What is the purpose of such double layers regulation? Is this translation regulation extendable to other structured 5'UTR mRNAs? Do p53 isoforms have the same effect?

MATERIAL AND METHODS

A. Cells and embryos.

HUVECs – Promocell – were cultured in endothelial growth medium – EGM-2 – containing 2% foetal bovine serum – Cambrex/Lonza – U87-MG human glioblastoma astrocytoma – n° ATCC : HTB-14, provided by LGC promochem, Molsheim, France – and fertilized chicken eggs – E.A.R.L. Morizeau, Dangers, France – were handled as described (Hagedorn *et al.* 2005). ABAE cells were grown in DMEM 10% Foetal bovine serum – FBS – with 2.5 mM L-Glutamine – From either Invitrogen, Paisley, UK or Lonza, Levallois- Perret, France – and FGF-2 at 2 ng/ml – From personal production. U2OS human osteosarcoma cell line – ATCC no. HTB-96 provided by LGC promochem, Molsheim, France – were grown in DMEM serum supplemented with 10% FBS and 2.5 mM L-Glutamine.

B. Transfections, transductions, drugs treatment and western blotting.

B.I. SiRNA and plasmid transfections in cells

Small interfering RNAs – siRNAs – were purchased from Eurogentec – Liege, Belgium – and Sigma-Aldrich – Saint-Quentin Fallavier, France. Sequences are provided in Sup Table II. SiNON served as a negative control. SiTAp53 targets the full TAD of p53 and SiΔ133p53 1 and 2 target the 5' untranslated region of Δ133p53. U87 and U2OS cells were transfected with the different siRNAs using InterferIN – Polyplus transfection, New York, USA – or with for plasmid transfection of p53 isoforms, using FuGENE reagent – Roche, Meylan, France.

B.II. Transductions

Following standard operating procedures, U2OS cell were transduced with replicating defective adenoviruses expressing p53 or the Green Fluorescent Protein – GFP at different multiplicities of infection. After six hours of treatment media were changed for fresh virus free medium. Transfection assessment was done by culture photos.

B.III. Drugs treatment and Western Blot

Actinomycin D and Doxorubicin – Sigma-Aldrich, York, UK – were at indicated titrations and for the time mentioned in the figure legends. When recovering cells for drug treatment, above mentioned media were used.

Western blots were performed as previously described (Bourdon *et al.* 2005). Primary antibodies were CM-1 and Sapu (Bourdon *et al.* 2005) for p53 and Δ133p53, respectively, 2A10 – Abcam, Cambridge, UK – for mdm2, TUB2.1 – Sigma-aldrich – for β-Tubulin and AC-15 – Sigma-aldrich – for β-Actin. Horseradish peroxidase-conjugated goat antibodies – Jackson ImmunoResearch, Baltimore – were used as secondary antibodies in immunoblots. Quantitative analysis of the immunoblot data was performed using the ImageJ 1.40g software.

C. Endothelial cell migration, tube formation and proliferation.

C.I. Wound Healing Assay

HUVEC and ABAE migrations in the "wounding assay" was performed respectively after eight and eighteen hours treatment with conditioned media from in siRNA-transfected U87 cells as described previously, i.e. after forty eight hours of siRNA treatment, cell media was changed to serum free medium for more twenty four hours (Bossard *et al.* 2004). Then newly formed conditioned media were transferred into the endothelial cell petri dishes.

C.II. Boyden Chamber assay

375 µL of U87 cells – 50×10^3/well – were seeded in 12 well plates and submitted to siRNA treatment as descrived above. One day after media were changed to serum free medium for one more day. Following manufacter's instructions inserts were added and 125 µL of ABAE cells – 100×10^3/insert – in DMEM with 2% FBS were deposited in the inserts allowing them to migrate for six hours. Staining with 1% Trypan Blue for thirty minutes was processed after removing the upper layer of ABAE cells with a cotton bud and fixation of the lower layer – migrating cells – with 4% formaldehyde for 15 minutes. Photos were taken with a stereomicroscope and cells counted with a software.

C.III. Matrigel

To assess the ability of cultured HUVECs to form vessel-like structures in culture – tube formation - Matrigel – BD Biosciences – was added to the wells of a 24-well plate in a volume of 300 µl and allowed to solidify at 37°C for 30 min. After the

Matrigel solidified, HUVECs – 50 × 10^3/well – were plated in 100 µl of media EGM-2 with 0.5 % serum and 300 µl of siRNA tumour cells transfected supernatant containing 20 ng/ml FGF2 final. Cultures were photographed and the mean number of branch points – SEM – per microscopic field was determined. Studies were performed in triplicate.

C.IV. Cell proliferation assay

Cell proliferation was determined by using the MTT [3-(4,5-dimethylthiaxol-2-yl)-2,5-diphenyltetrazolium bromide] assay. HUVECs – 2 × 10^3/well – were incubated with EBM-2 medium with 2 % FBS in 96-well plates. ABAE cells – 15 × 10^3/well – were incubated with DMEM medium with 2 % FBS in 96-well plates. U87 cells – 5 × 10^3/well – were incubated as described above. HUVECs and ABAE were serum starved overnight and incubated in 50 µl of rerspectively media EGM-2 with 2 % serum or DMEM with 2 % serum and 150 µl of siRNA tumour cells transfected supernatant containing 3 ng/ml FGF2 final. The medium then was aspirated and MTT was added to each well – 0.25 mg/ml. Cells then were incubated for a further 4 h at 37°C. The medium then was aspirated and the cells were lysed with DMSO and absorbance at 540 nm was measured.

C.V. ELISA

FGF-2 ELISA kits were purchased at Biolegends, supplier Ozyme, Saint Quentin Yvelines, France. Conditioned media from SiRNA treated U87 cells were used to assess FGF-2 secretion. Manufacturer's procedures were followed and experiment repeated twice.

C. Tumour in CAM and xenografts in mouse.

SiRNA transfected U87 cells were deposited on CAM as described (Hagedorn *et al.* 2005). 18 Digital photos were taken on Day 4 using a stereomicroscope – Nikon SMZ800.

For xenografts, were injected subcutaneously into nude background – n=8. Animals were sacrificed ten days later. Six-week-old nude mice were purchased from Janvier. Mice were injected with 10^6 siRNA transfected U87 cells subcutaneously into

the mid-back region. The tumour size was measured in three dimensions with calipers one time a week starting at Day 7. Mice were observed for any change in behavior, appearance or weight. At the end of the experiment at Day 10 and Day 44, mice were killed and xenograft specimens were harvested for further analyses.

Tumours were considered as oblate half spheroids for the estimation of the volume using a stereomiscroscope and abode Photoshop software.

D. Histology and immunochemistry.

Tumours from CAM xenografts were fixed and cryo-sectioned as described (Saidi *et al.* 2008). Ten micrometer sections were stained with haematoxylin and eosin. For immunohistology studies, the following primary antibodies were used: Ab-2 – clone V9, NeoMarkers Ab, Montluçon, France – for Vimentin, Anti Ki-67 – AnaSpec Inc., Fremont, CA – for Ki-67, Fluorescein-coupled SNA-1 – AbCys, Paris, France – for Sambucus Nigra Lectin (SNA-lectin). Cell nuclei were visualized by DAPI dye. Corresponding secondary antibodies were from Molecular Probes – Invitrogen. SNA-lectin staining allowed quantification of the capillary network invading the tumour by counting the number of green pixels on each field with Adobe Photoshop software. The whole surface of tumours has been counted because of capillary staining heterogeneity – at least 5 tumours / group.

For mice tumour xenografts, five thick cryosections of tissues were prepared using a Leica cryostat CM3050. Slides were fixed in ice cold acetone for two minutes, permeabilized in 0.1% Triton X-100 in phosphate buffered saline – PBS – blocked in 5% bovine serum albumin in PBS for 1 hour at room temperature and then incubated with 5 µg/ml primary antibodies for 1 hour at room temperature. Blood vessels were detected with 5 µg/ml anti-CD31 – BD Pharmingen – antibody. After extensive washing, slides were incubated with 1-2 µg/ml cross-absorbed goat anti-rat DyLight 549 – Tebu-bio – secondary antibody for 1 hour at room temperature. Slides were counterstained with DAPI – Tebu-bio. Coverslips were mounted with Dako Cytomation fluorescent mounting medium – Dako. For quantification, number of blood vessels in 5-10 microscopic fields per cryosection – per animal – was quantified and the mean number of vessels +/- s.e.m. for the entire treatment group determined.

E. RNA extraction, nested RT PCR, real time qRT-PCR and TLDA.

Total RNA was isolated from U87 cells using Nucleospin RNA II kit – Macherey Nagel, Hoerdt, France – assessed and nested RT PCR was performed as described previously (Bourdon *et al.* 2005).

Quantitative PCR was performed on StepOne+ – Applied Biosystems – using 10 to 100 ng of cDNA and Power SYBER Green or Taqman Universal PCR master mix – Applied Biosystems – for detection of FLp53, Δ133p53 and 18S.

Taqman low density array – Applied Biosystems – was performed following manufacturer instructions with 50 ng of cDNA. The results were treated with the StepOne Software v2.0 – Applied Biosystems. Primers sequences are provided in Sup Table II.

F. Plasmid constructions

Δ133p53α, Δ133p53β and Δ133p53γ plasmids for cloning experiments were obtained from Heiley Moore, SuMO laboratory, Dundee and the pTRIP-TRE-Tight-MCS plasmid was obtained form the A. Delluc, BiViC platform, Toulouse. Restriction enzymes and ligases were from New England Biolabs, suppier Ozyme, Saint Quentin Yvelines, France. Fragment and Plasmid purification kits were from Macherey Nagel, Hoerdt, France.

G. Luminoter assays

Luminoter experiments were done following Promega's procedures. 100 µL/well of 1X Passive lysis Buffer were added to the U2OS stable cell lines for 15 minutes and homogenized and transferred to plain white 96 well/plates. Luciferases – dual luciferases, Promega, Charbonnières-les-Bains, France – addition and measurement – 30 µL/luciferase for 10 sec – were done by a Berthold Luminometer – Berthold, Thoiry, France.

H. Statistical analysis

All statistical determination was performed with GraphPad Prism, version 4.0 – GraphPad, San Diego, CA. All data are presented as the mean ± SEM. One-way and two-way analysis of variance was used to evaluate the significance of differences between groups. All statistical analyses were performed with a two-tailed Student's t-test. A p value of less than 0.05 was considered statistically significant.

RESULTS

Résumé de la partie résultats

Suite à la découverte et la caractérisation de 9 nouvelles isoformes du gène de p53, J'ai focalisé mon travail sur les isoformes dépourvues du domaine de transactivation : les isoformes Δ133p53. Plus particulièrement j'ai étudié l'implication de ces isoformes lors de l'angiogenèse et la progression tumorale, qui m'ont permis de mettre en évidence leu rôle activateur prépondérant. En effet par une approche d'ARNi j'ai pu démontrer que lorsque l'on inhibait l'expression des Δ133p53 dans des glioblastomes humains possédant une p53 sauvage, les tumeurs présentaient une absence de réseau angiogénique *in vivo* dans un modèle de CAM. De la même manière j'ai démontré dans un modèle murin immunodéficient xénogréffé une diminution nette du nombre de vaisseaux, ainsi qu'une croissance tumorale plus lente. Ce mécanisme vis-à-vis de l'angiogenèse résulte d'une inhibition de la migration et la tubulogenèse des cellules endothéliales, et non de leur prolifération. Au contraire les cellules tumorales traitées par les SiΔ133p53 prolifèrent beaucoup moins rapidement que les mêmes cellules contrôles. Grace à une étude large spectre de la transcription de facteurs impliqués dans l'angiogenèse, J'ai identifié plusieurs cibles, l'Angiogenine, la midkine, IL-12α, ANGPTL4 et HGF régulées par Δ133p53. Cette étude nous a également permis d'identifier des cibles spécifiques de Δ133p53, ce qui n'a jamais été encore démontré, et de nouveaux gènes cibles de p53 comme la pléiotrophine et le VEGF-C. Enfin des lentivecteurs exprimant des isoformes Δ133p53 que j'ai construit sont produits afin d'étudier finement l'impact de leur surexpression.

Je me suis également penché sur le mode de régulation du FGF-2 par p53. Les études précédentes dans notre laboratoire et ailleurs montrent un système complexe de régulation. Sur une lignée d'ostéosarcome humain, par approche ARNi, j'ai pu démontrer que la p53 endogène était capable d'inhiber l'activité IRES du FGF-2 et de C-myc. Au contraire j'ai constaté que p53 ne modifiait pas l'expression de l'ARNm du FGF-2, ni sa présence dans le milieu extracellulaire. A la suite de stress génotoxique tel que la doxorubicine, j'ai montré que le FGF-2 endogène subissait une diminution transitoire de sa présence protéique sans subir de variation de son expression de son messager. En revanche une forte surexpression de p53 par adénovirus ne modifie par l'expression du FGF-2, suggérant une modification post-traductionnelle spécifique de p53.

Pour finir, j'ai participé aux travaux de Moustapha Aoubala sur l'étude de la surexpression de l'isoforme Δ133p53 en réponse au traitement à la doxorubicine, dans des lignées de glioblastomes humains (Aoubala *et al.* 2010).

CHAPTER 1:

Involvement of the Δ133p53 isoforms in angiogenesis and tumour progression in a glioblastoma model

Δ133p53 isoforms which include in this nomination α, β and γ isoforms – respectively bearing 261, 209 and 214 a.a. – do not contain the p53 TAD in the N-terminus domain. They even lack a part of the first of five well conserved DBDs. For this reason, it is widely suggested that Δ133p53 isoforms do not have transcriptional activity and modulate p53 activity through the binding of p53 when associating in a tetramer form. The β and γ spliced isoforms do not contain the C-terminal oligomerization domain and in exchange possess 11 and 16 a.a. sequences. Our study showed that the full length p53β isoform does bind p53 indicating that the β splice still may confer the ability to tetramerize (Bourdon *et al.* 2005). Additionally, it is however reasonable to question the efficiency of inhibition regarding the quantity of bound N-truncated isoforms to p53. Indeed, Chan *et al.* established that only one N-truncated molecule of p53 is needed to inhibit the transcriptional activity of a tetramer and DNA binding is not necessary for these isoforms to inhibit p53 (Chan *et al.* 2004). Surprisingly, three molecules of mutant p53 are necessary to inhibit the p53 tetramer. Consequently, even if these truncated isoforms are less expressed than the full length p53, they still conserve the ability to totally inhibit p53.

Δ133p53 isoforms unlike full length p53 are not expressed in all normal tissues. Δ133p53α is expressed in 14 tissues over 18 tested, Δ133p53β in 6 and Δ133p53γ in 10. For instance in breast tissues none of the short isoforms are expressed; in colon, bone marrow, spleen and testis all isoforms are expressed. Although we did not elucidate the reason of these differences, it would explain in part why p53 does not respond equally and does not trigger a response with same amplitude in different tissues (Lindsay *et al.* 2007).

So far, Δ133p53 is considered as dominant negative of p53 through binding of p53 and inhibiting its transcriptional activity. Based on the studies mentioned above, an alteration of Δ133p53 expression would result on an inhibition of p53 related induction of apoptosis and senescence. Particularly, and because of the localisation of Δ133p53 in the nucleus and the cytoplasm, the short isoform would be able to inhibit this pathway and would block interaction of p53 and its cytoplasmic partners – Bcl-2 for instance – for mitochondrial associated apoptosis.

Another important aspect of Δ133p53 isoforms is their expression in abnormal cells like cancer cells. As we first showed Δ133p53 isoforms are differentially expressed in cancer cells and overexpressed in breast cancer (Bourdon *et al.* 2005). These observations have been confirmed in other cancer types I have previously

mentioned in this report: colon cancer, melanoma and renal cancer. These studies highlight the importance of two isoforms mainly, Δ133p53 and p53β. To note Horikawa *et al.* in 1995 discovered a p53 mutant isoform in human choriocarcinoma cell line CC1 resulting from mRNA lacking exon 2, 3 and 4… and starting at codon 133! Nevertheless, they didn't compare it with normal cell lines (Horikawa *et al.* 1995).

Shorty after the discovery of the p53 isoforms, I investigated different aspects of Δ133p53. Among them I wanted to see whether these isoforms are able to modulate the migration of cancer cells that are wildtype for p53. The laboratory of Pierre Roux has strong expertise in mechanisms involving p53 related migration of cancer cells (Roger *et al.* 2006). They did some preliminary experiments in MEF cell line overexpresssing each Δ133p53 and found that these isoforms were able to induce the motility of the cells – data not shown. As invasion and angiogenesis are strongly related through different molecular events such as remodelling of the extra-cellular matrix –ECM – and loosening of cell to cell interaction, these results encouraged us to start a new project involving the role of Δ133p53 isoforms in angiogenesis.

The choice of the glioblastoma model U87 – also known as U87 MG – was directed by the features of this cell line: we needed a wild type p53 cancerous cell line, a cell line that induces strong angiogenic response, a model that would allow to separate tumour growth and angiogenesis and in which this cell line has been properly studied. Consequently, the chorioallantoic membrane model became clearly the one of interest and the p53 wildtype U87 MG – U87 – cell line appeared to be the most convenient of these studies.

The well-known tumour suppressor p53 qualified of "the guardian of the genome" is mainly described as a single protein. However we have shown that the p53 gene exhibits a complex pattern of expression leading to synthesis of nine isoforms (Bourdon *et al.* 2005; Bourdon 2007). Alternative splicing of intron 9 generates p53 isoforms bearing different C-terminal domains – α, β and γ – Figure 23A. Initiation of translation at an alternative AUG codon leads to Δ40p53 isoforms devoid of part of the TAD, whereas the presence of an alternative promoter in intron 4 generates Δ133p53 isoforms lacking the TAD and part of the DNA-binding domain (Yin *et al.* 2002; Bourdon *et al.* 2005). P53 isoforms are conserved from Drosophila and zebrafish to human, but little is known about their functions (Bourdon *et al.* 2005; Chen *et al.* 2009). Overexpression of Δ133p53 in human breast tumours versus normal breast tissue suggests a protumoural involvement of these isoforms, contrasting with the tumour suppressor activity of p53 (Bourdon *et al.* 2005). Indeed, human Δ133p53 as well as its zebrafish ortholog is able to antagonize wild-type p53 pro-apoptotic function (Chen *et al.* 2009). Furthermore, increased expression of Δ133p53 is associated with a loss of senescence (Fujita *et al.* 2009).

In addition to its ability to control DNA repair, cell cycle arrest and apoptosis, p53 is involved in inhibition of angiogenesis, a critical mechanism in tumour progression and metastatic dissemination (Teodoro *et al.* 2007). This process is induced in most solid tumours, whose centers become hypoxic following an increase of the diffusion distance between the nutritive blood vessels and tumour cells. Hypoxia is one of the principal angiogenic stimuli leading to synthesis of angiogenic growth factors and inducing formation of new blood vessels (Pouyssegur *et al.* 2006; Fraisl *et al.* 2009). Such vessels, in addition to their ability to feed tumour cells, provide a way for cells to disseminate and form metastases. p53 inhibits angiogenesis by at least three mechanisms:

1) by interacting with the central regulator of hypoxia, the hypoxia-induced transcription factor HIF1-α,

2) by inhibiting production of pro-angiogenic factors such as vascular endothelial growth factor (VEGF) and fibroblast growth factor 2 (FGF2), and

3) by increasing production of anti-angiogenic factors (Dameron *et al.* 1994; Ueba *et al.* 1994; Ravi *et al.* 2000; Galy *et al.* 2001; Pal *et al.* 2001; Folkman 2006; Teodoro *et al.* 2007).

p53 is able to induce tumour dormancy, and to block or reverse the tumour switch from dormant non-angiogenic state to angiogenic invasive phenotype (Giuriato *et al.* 2006; Naumov *et al.* 2006). Clinical data also suggest that p53 plays a crucial role in controlling tumour vascularization. However, nothing is known about the role of Δ133 isoforms in this process.

Among all solid tumours, glioblastoma multiform – GBM – are the most angiogenic by displaying the highest degree of vascular proliferation and endothelial cell hyperplasia (for review (Wong *et al.* 2009)). Such intense vascularization plays a critical role in the pathological features of GBM, including peritumoural oedema resulting from the defective blood brain barrier – BBB – in the newly formed vasculature. Thus, human glioblastoma U87, expressing wild type p53, has been chosen here to address the role of Δ133p53 in angiogenesis and tumour progression.

In the present study we show by a knockdown approach that Δ133p53, in contrast to p53, is necessary for endothelial cells migration and tube formation *in vitro*, whereas it allows angiogenesis and tumour progression *in vivo*. Taqman Low Angiogenesis Array analysis showed that Δ133p53 pro-angiogenic activity is associated with a specific modulation of the angiogenic balance.

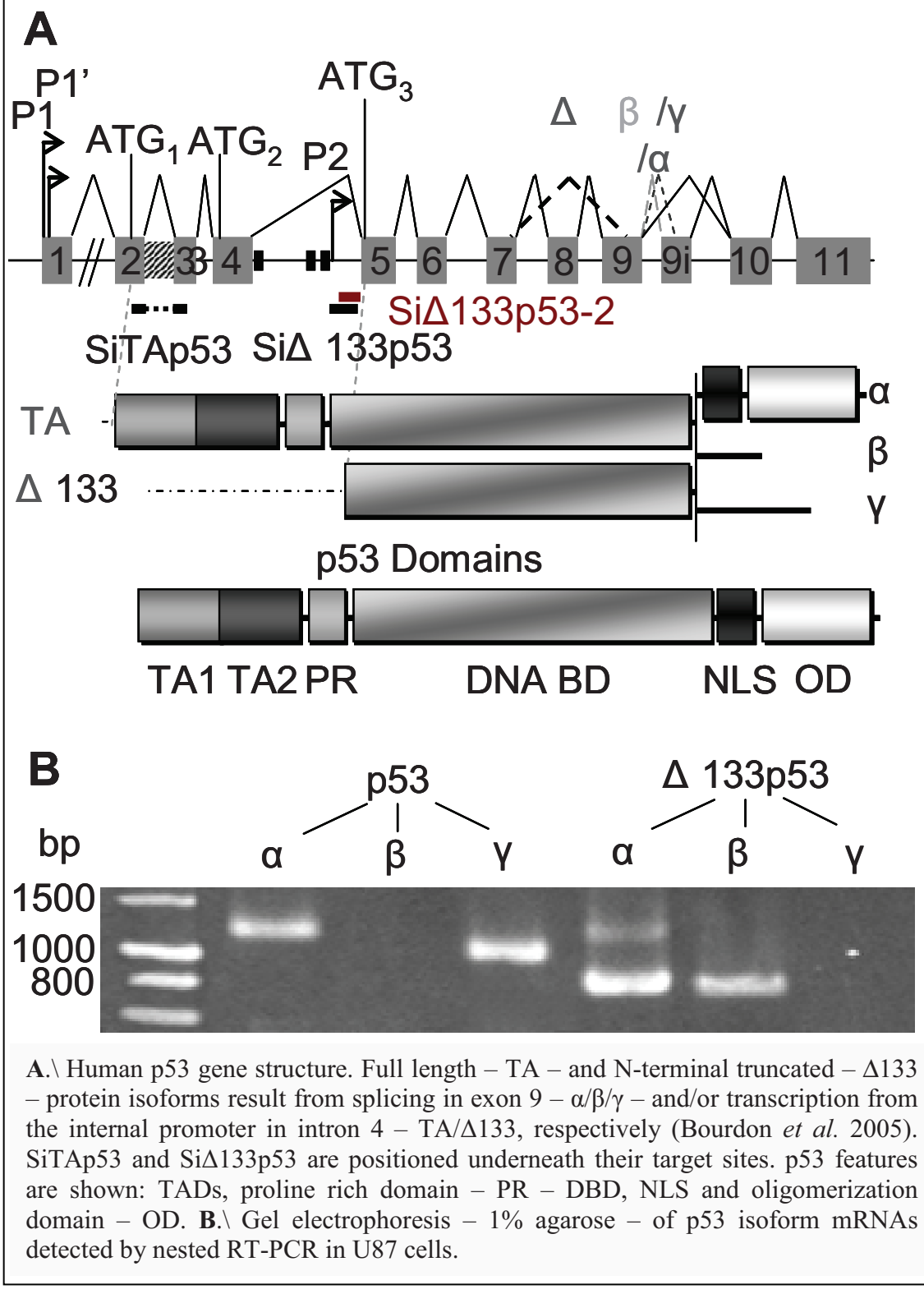
A. Δ133P53 knockdown activates p53 expression.

p53 isoforms expression was analysed by nested RT PCR in U87 human glioblastoma, known to exhibit a wild type p53 genotype – Figure 23B. This allowed us to detect p53, p53γ, Δ133p53 and Δ133p53β, but not p53β and Δ133p53γ. To note, In comparison with human normal brain tissue, U87 cell lines express the Δ133p53β isoform (Bourdon *et al.* 2005). Also of important and maybe relevant for embryo development, human foetal brain tissues express all p53 isoforms evaluated but not Δ133p53α.

U87 cells were treated by siRNAs targeting either p53 or Δ133p53, named SiTAp53 and SiΔ133p53, respectively – Figure 23A. Knockdown efficiency was analysed by qRT-PCR – Figure 23C. Results showed that SiTAp53 and SiΔ133p53 generate specific knockdown of the targeted isoforms compared to the control SiNON. This was confirmed by Western blot – Figure 23D and E. Unexpectedly, Δ133p53 knockdown generated an increase of p53 expression visible at both protein and mRNA levels, also reflected by up-regulation of the p53 target mdm2 Figure 23C and D.

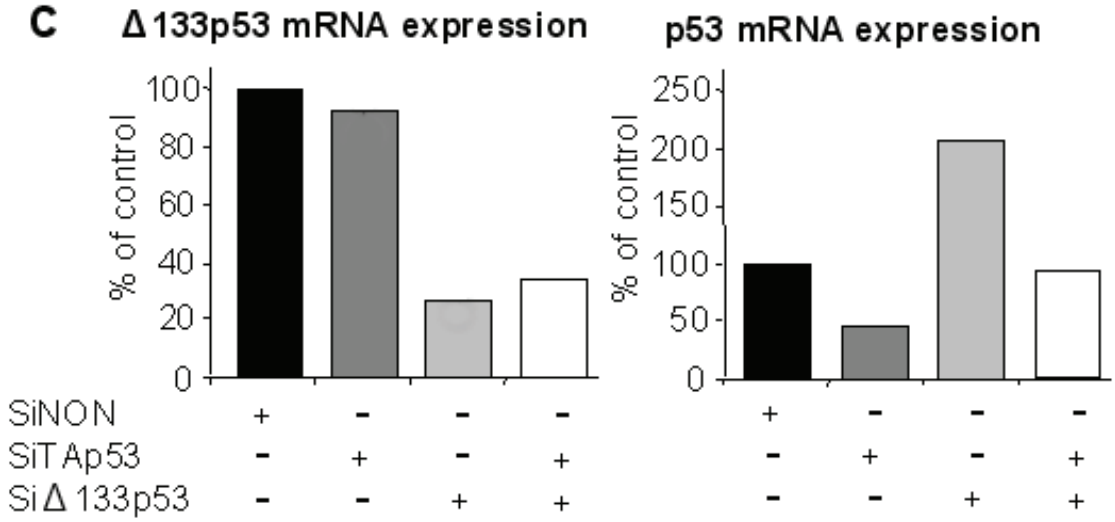
These data validated SiTAp53 and SiΔ133p53 as efficient tools to knockdown p53 and Δ133p53, respectively. In addition, they also suggest that Δ133p53 might inhibit p53 expression.

Figure 23. Expression and knockdown of p53 isoforms in U87 cells.

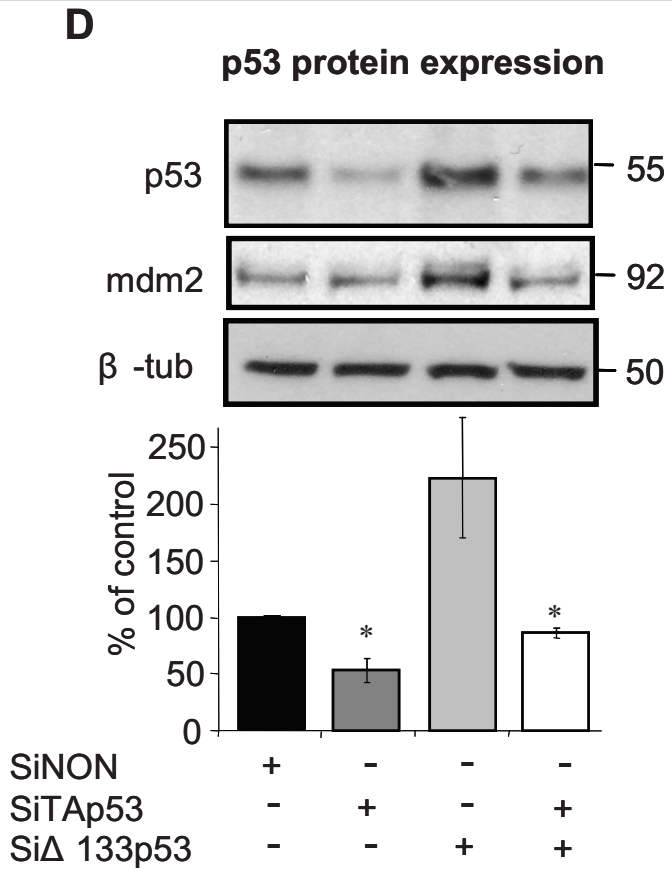


A.\ Human p53 gene structure. Full length – TA – and N-terminal truncated – Δ133 – protein isoforms result from splicing in exon 9 – α/β/γ – and/or transcription from the internal promoter in intron 4 – TA/Δ133, respectively (Bourdon *et al.* 2005). SiTAp53 and SiΔ133p53 are positioned underneath their target sites. p53 features are shown: TADs, proline rich domain – PR – DBD, NLS and oligomerization domain – OD. **B**.\ Gel electrophoresis – 1% agarose – of p53 isoform mRNAs detected by nested RT-PCR in U87 cells.

Figure 23. Expression and knockdown of p53 isoforms in U87 cells.

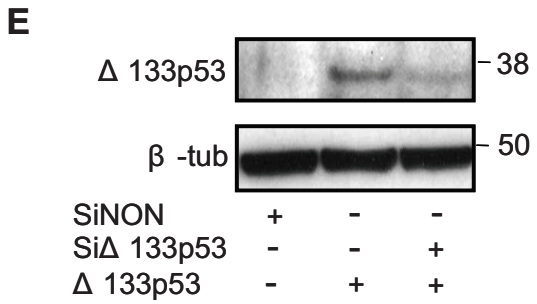


C.\ Δ133p53 – left – and p53 mRNAs – right – relative expression following siRNA treatment – qRT-PCR. Experiments were repeated twice in duplicate and mean is represented. Sample quantity is normalized with 18S rRNA using the $2^{-\Delta\Delta Ct}$ method and set to percentage of control.



D.\ p53 and mdm2 – p53 target – expression analysed by Western blot following siRNA treatments – β-tubulin used as a control. Experiments were repeated four times for p53 detection and twice for mdm2. The histogram shows p53 signal quantification from Western blot – 4 independent experiments.*, $p<0.05$. vs SiNON control group. Statistics done sith Student test. p53 immunostaining with Sapu antibody, mdm2 is with 2A10 antibody and β-tub with β-tubulin antibody

Figure 23. Expression and knockdown of p53 isoforms in U87 cells.

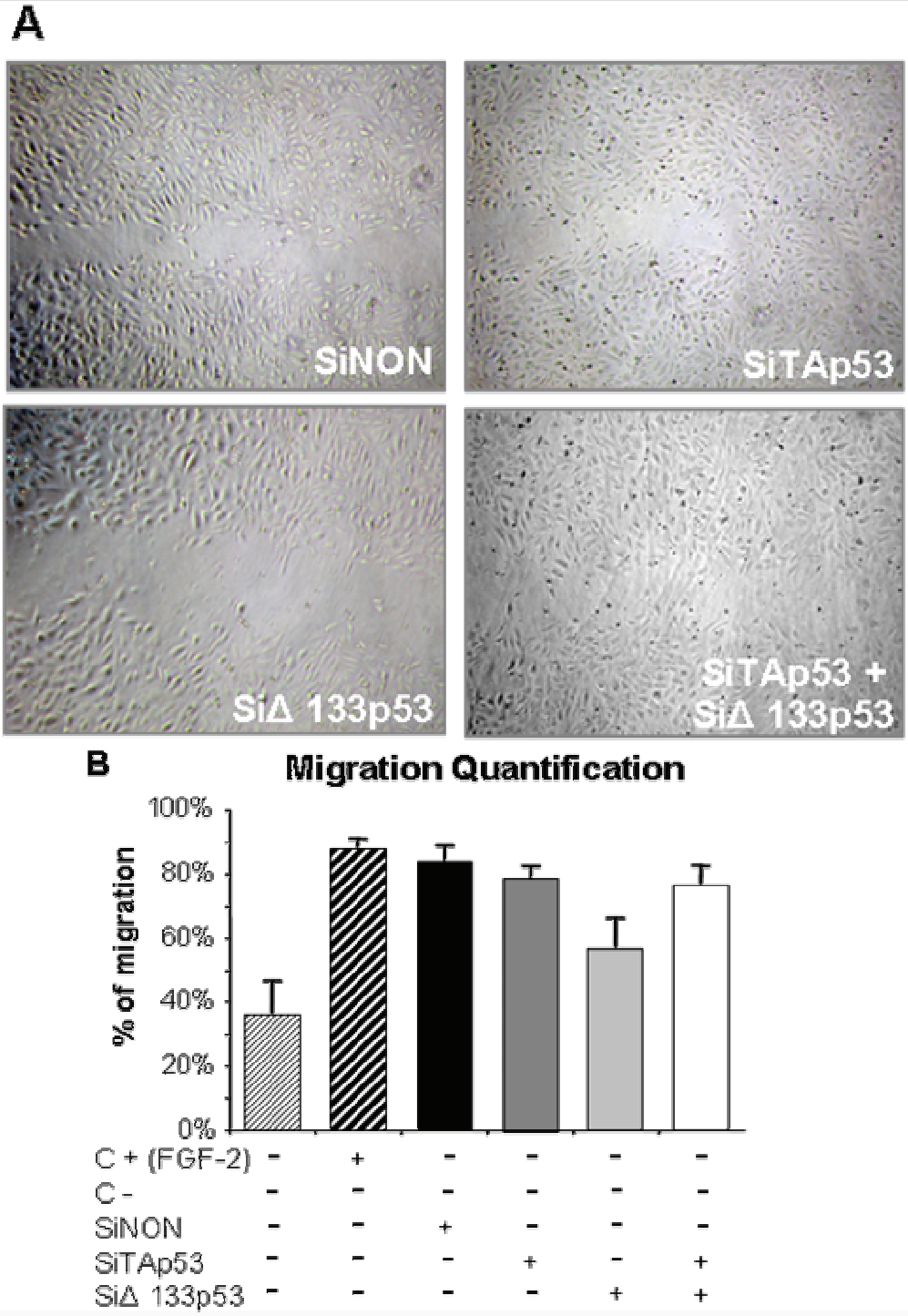


E. Δ133p53 protein expression in U87 folowing siRNA treatment and Δ133p53 overexpressino by plasmid transfection – Western blot. Experiment were repeated twice. Δ133p53 immunostaining with Sapu antibody and β-tub with β-tubulin antibody

B. Δ133P53 knockdown blocks endothelial cell migration and tubulogenesis.

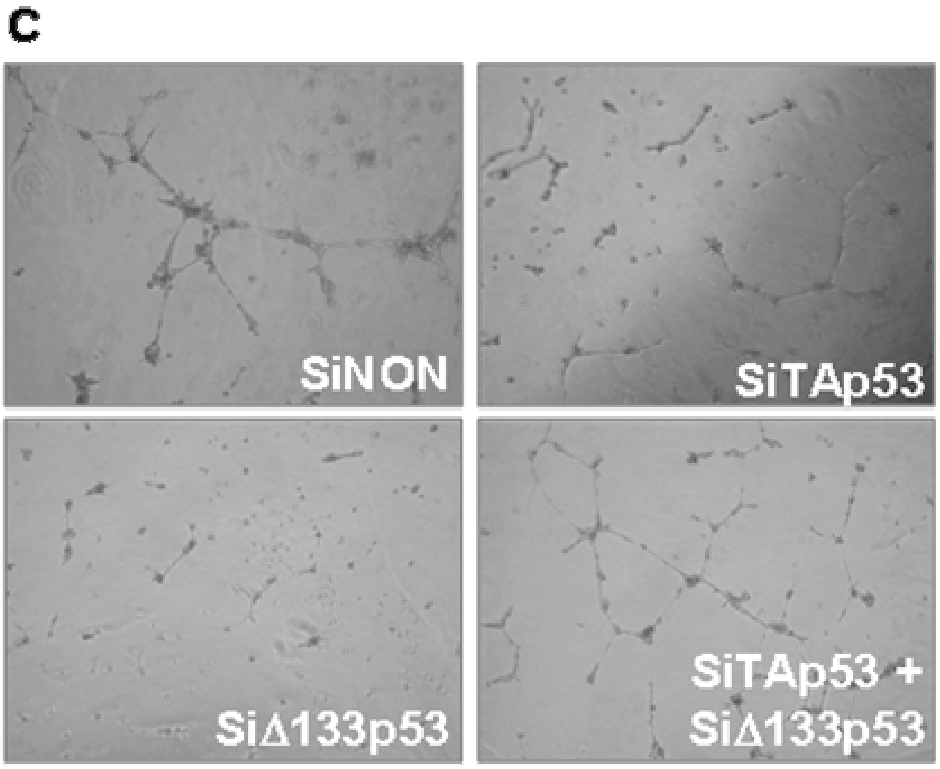
Anti-angiogenic features reported for p53 prompted us to look at the effect of Δ133P53 in this process. This issue was first addressed *in vitro*, using human umbilical vein endothelial cells – HUVECs. HUVECs were treated with conditioned media produced by siRNA-transfected U87 cells, and their ability to migrate was analysed by the classical wound assay – Figure 24A and B. Data clearly showed that SiΔ133P53 inhibits HUVECs migration. Tube formation in matrigel was also significantly inhibited by SiΔ133P53 treatment – Figure 24C and D – whereas HUVECS proliferation was not affected – Figure 24E. The blockade of HUVEC migration and tube formation was abolished in the double knockdown by SiTAp53 + SiΔ133P53 – Figure 24B and D – indicating that Δ133P53 is employed in normal endothelial cell migration and tube formation by a mechanism that might involve a dominant-negative effect on p53 activity.

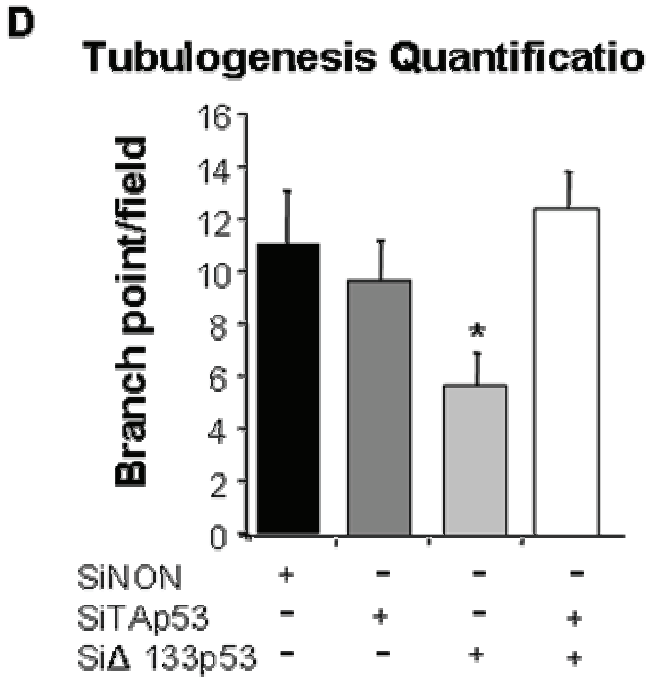
Figure 24. Δ133P53 knockdown blocks HUVEC migration and tube formation, but not proliferation.



A-E.\ Conditioned medium from knocked-down U87 were used to assess HUVEC migration, tube formation and proliferation. **A**.\ Micrographs of scratch wound healing assay of HUVEC in the presence 10ng/mL FGF2-contatining supernatants of U87 cells transfected with siRNAs as indicated. **B**.\ Quantification of HUVEC migration in scratch wound area.Asterisk indicates statistical significance. *, $P < 0.05$ vs the control SiNON-treated group. Statistics done with Student test.

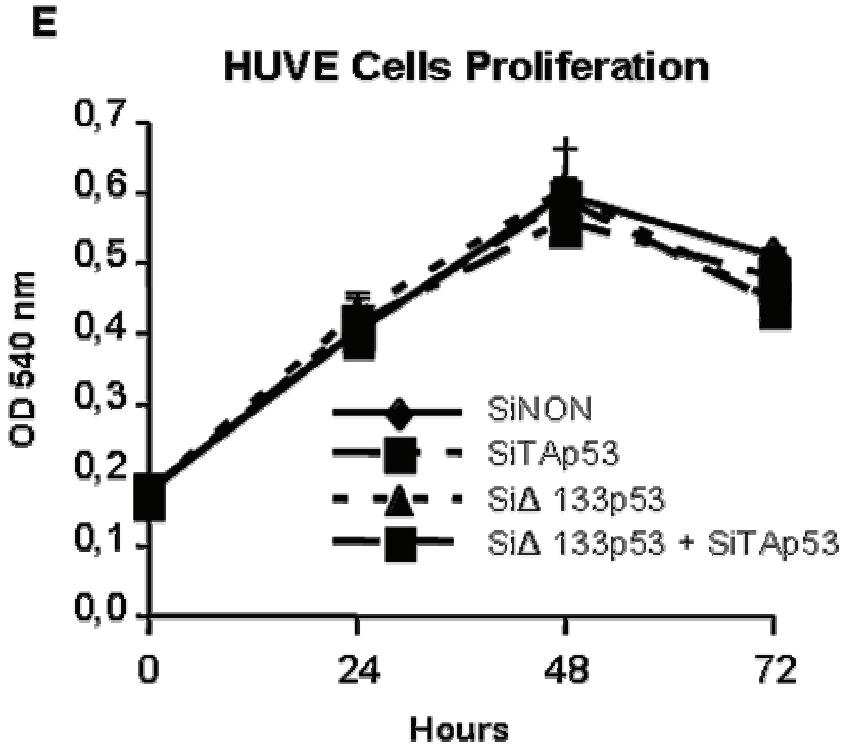
Figure 24. Δ133P53 knockdown blocks HUVEC migration and tube formation, but not proliferation.





C.\ Micrographs of tube formation by HUVEC in Matrigel containing 10 ng/mL FGF2 in the presence of U87 cells supernatants. **D**.\ Quantification of the number of branch points formed. Asterisk indicates statistical significance. *, $P < 0.05$ vs the control siNON-treated group. Statistics done sith Student test.

Figure 24. Δ133P53 knockdown blocks HUVEC migration and tube formation, but not proliferation.



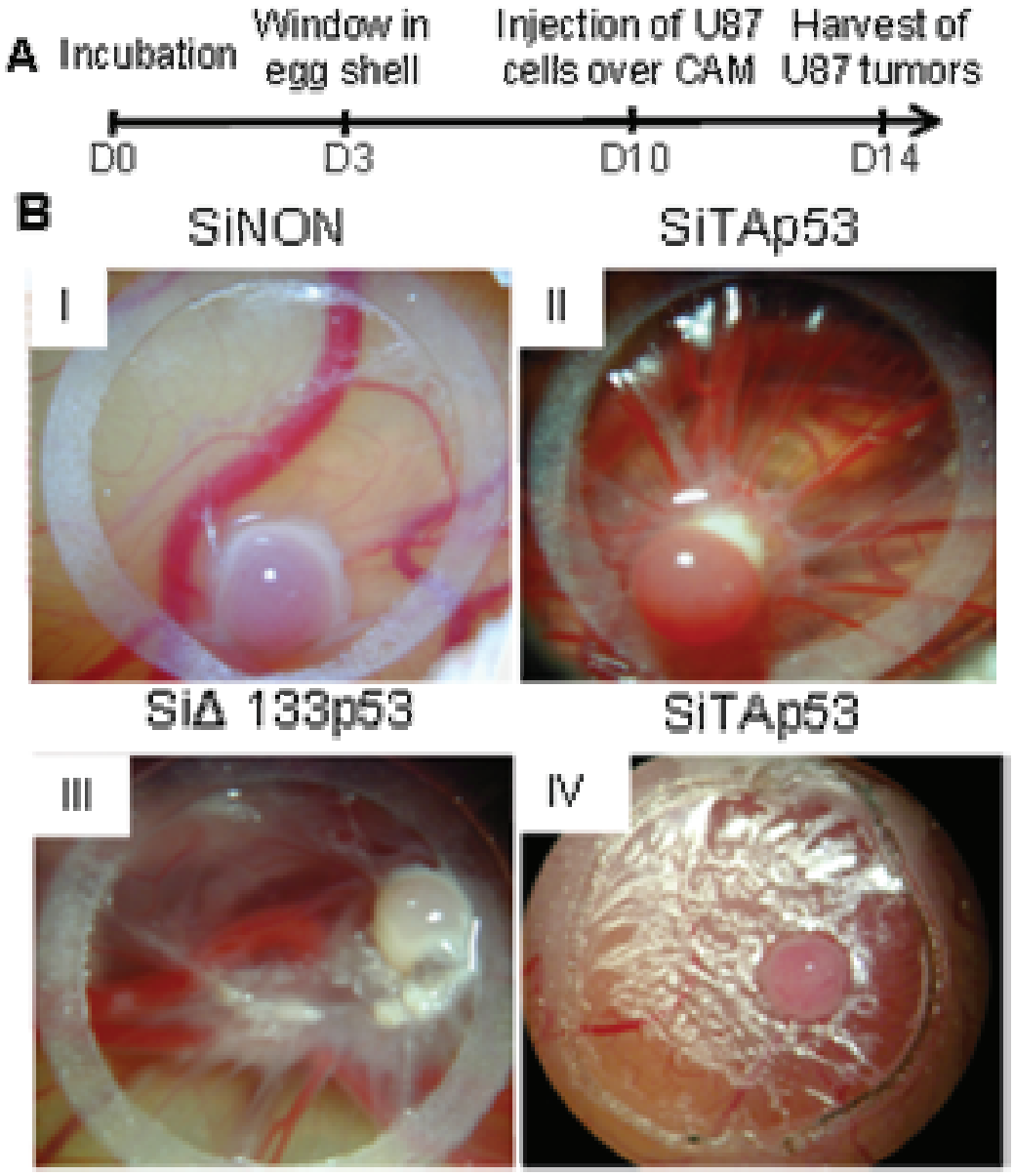
E.\ Effects of siRNA-transfected U87 cells supernatants containing 3ng/mL FGF2 on HUVEC proliferation. HUVECs were incubated with supernatants for 24, 48 and 72 h. Cell proliferation was determined by the MTT assay.

C. □133P53 knockdown is anti-angiogenic and prevents tumour growth in CAM.

Δ133P53 effect on angiogenesis and tumour growth was addressed *in vivo* using the experimental glioma assay developed on the chicken chorio-allantoic membrane – CAM – as described previously (Hagedorn *et al.* 2005). This experimental glioma recapitulates hallmarks of human GBM and allows following the first steps of tumoural angiogenesis. SiRNA-transfected U87 cells were deposited on the CAM of fertilized eggs and tumours were analysed 4 days later – Figure 25A. SiTAp53 and SiΔ133P53 exhibited opposite effects on tumour angiogenesis: p53 knockdown resulted in increase of tumour vascularization, whereas Δ133p53 knockdown generated avascular tumours, showing that it strongly inhibited angiogenesis – Figure 25B and C. Angiogenesis inhibition by SiΔ133P53 was confirmed by SNA-lectin quantification of vessels – Figure 25D. Such anti-angiogenic effect of SiΔ133P53 was also correlated with smaller size of tumours at day 4 – Figure 25E. Western blot confirmed that siRNAs were still efficient in tumours – Figure 25, F and G. Interestingly, tumours resulting from the double knockdown by SiTAp53 and SiΔ133p53 were similar to the control, as observed in vitro.

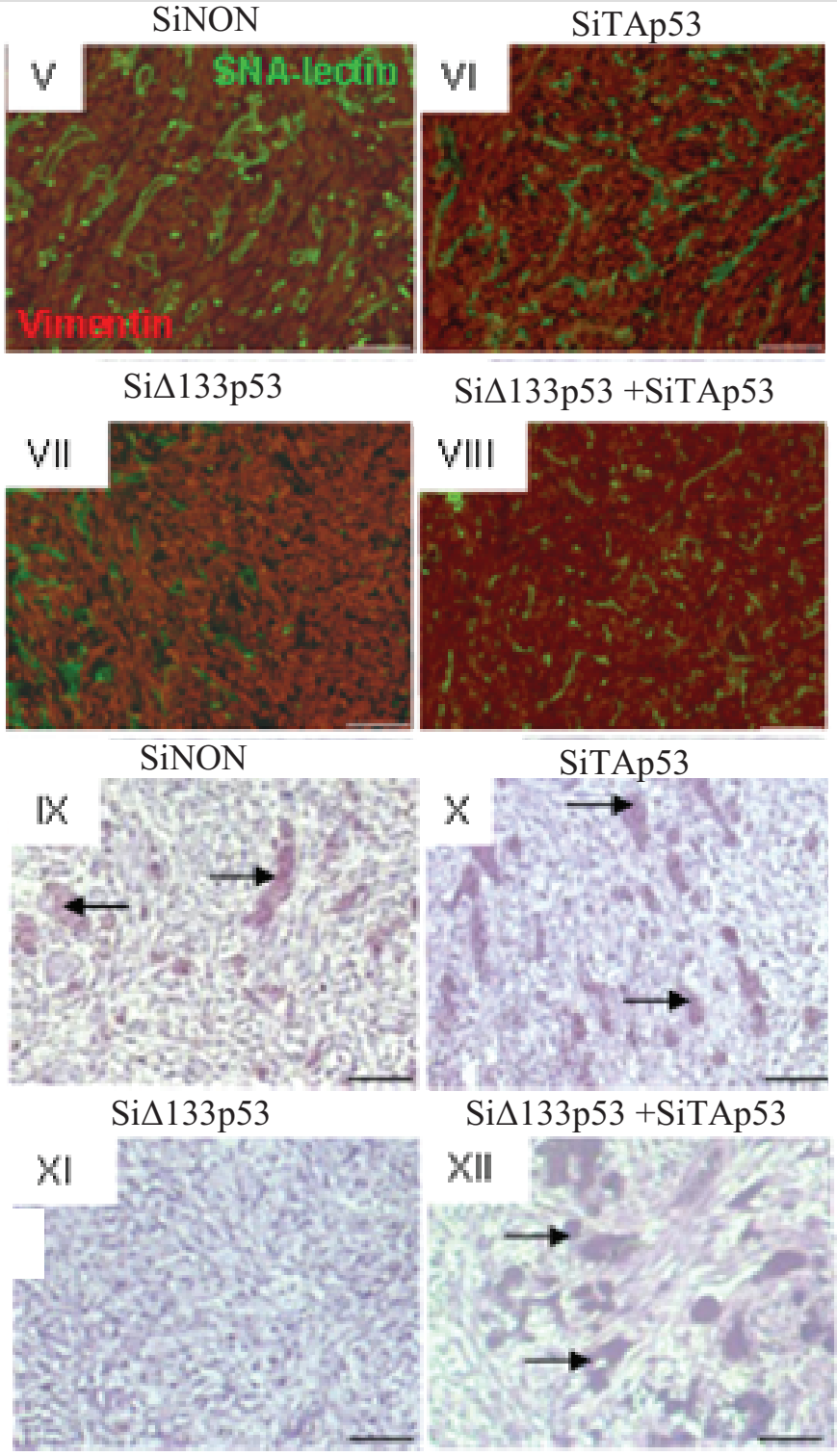
These results strongly suggested that Δ133p53 might exhibit pro-angiogenic and tumour accelerating activities, indicating that p53 and Δ133p53 might antagonize each other in the angiogenic conversion of glioblastoma.

Figure 25. Δ133P53 knockdown prevents tumour growth and angiogenesis in CAM.



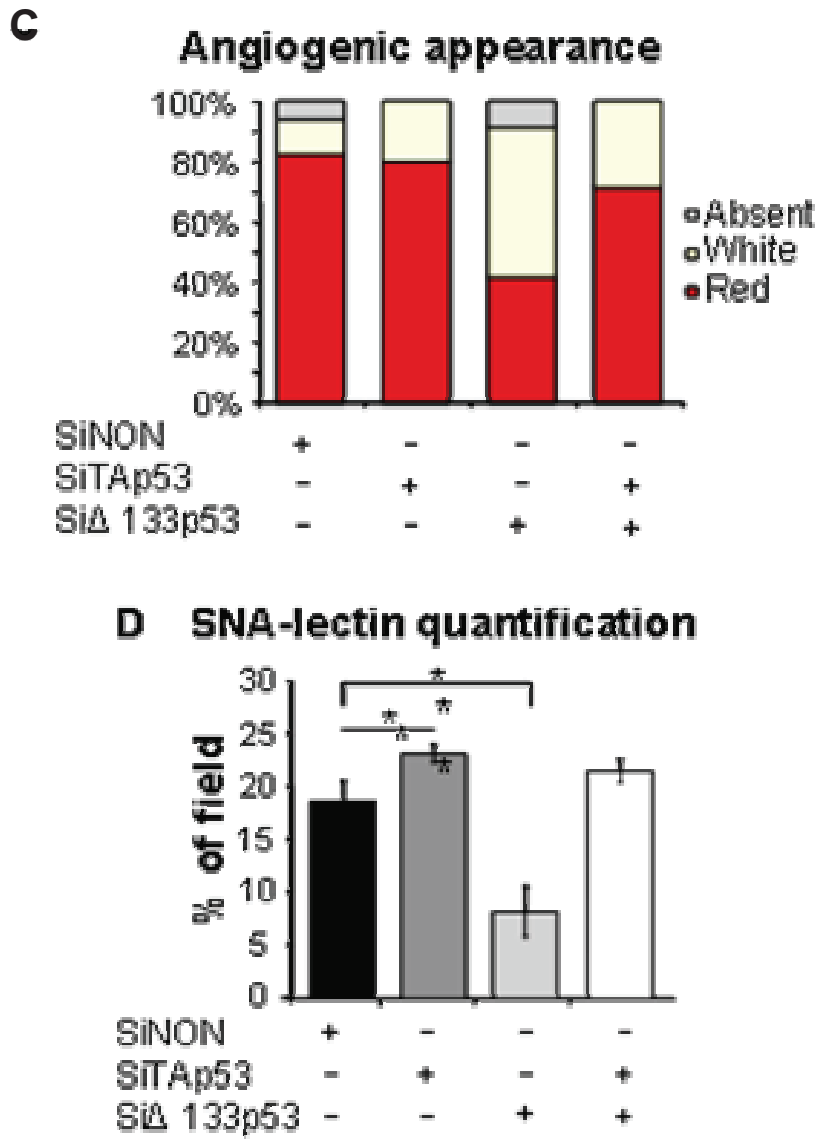
U87 tumours were analysed in CAM following knockdown of p53 and/or Δ133p53. **A**.\ Experimental protocol. **B**.\ Histological analysis of p53 and Δ133p53 knockdown in U87 tumours 4 days after implantation. I, II, III and IV.\ Tumour growth was assessed by biomicroscopy – ×3 magnification,

Figure 25. Δ133P53 knockdown prevents tumour growth and angiogenesis in CAM.



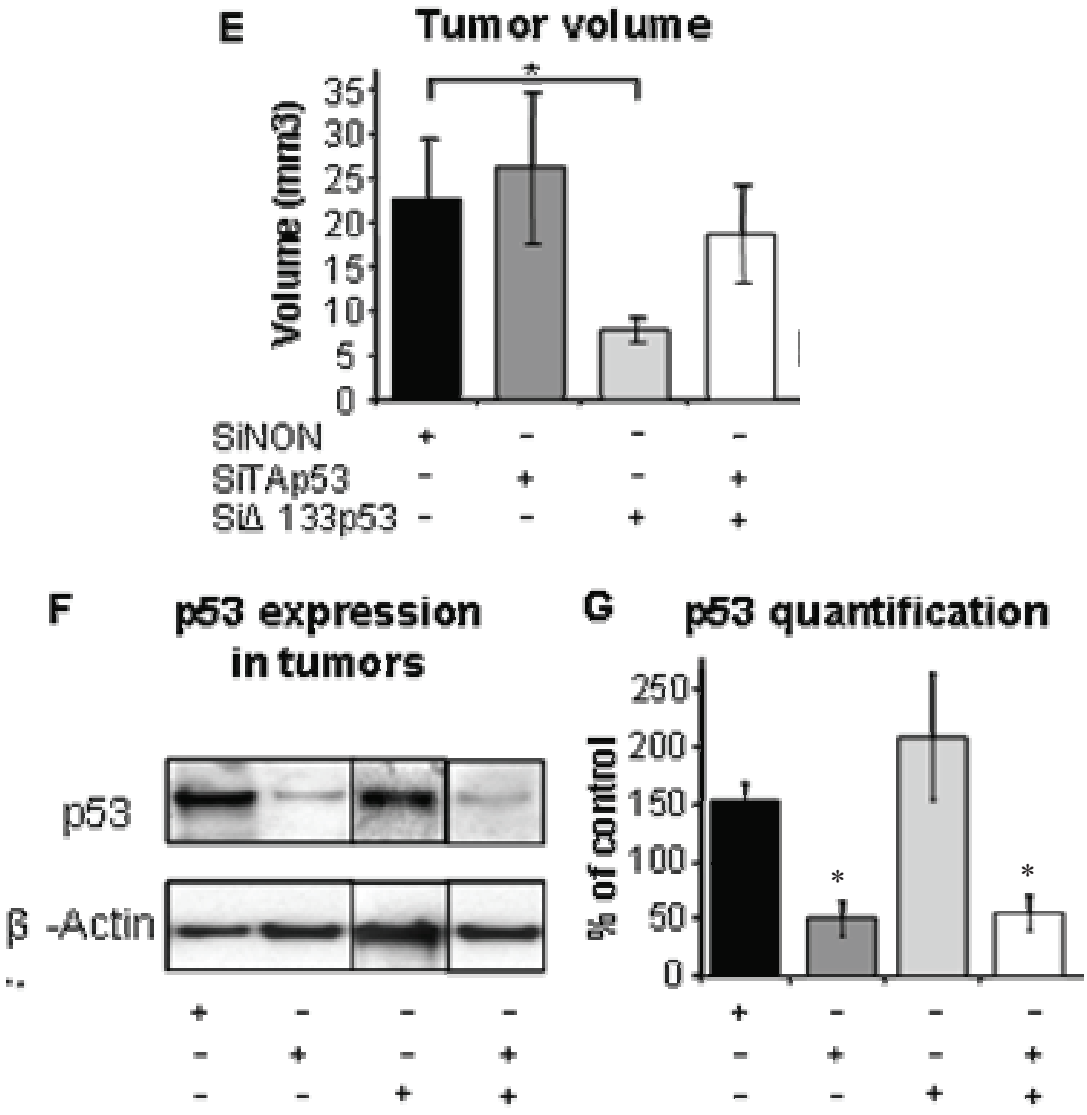
Histological analysis of p53 and Δ133p53 knockdown in U87 tumours 4 days after implantation. V, VI, VII and VIII.\ immunohistology – x20 magnification – and IX, X, XI and XII.\ H&E histology – x20 magnification. Images were taken in the center of the tumour. Tumour undifferentiated state and pro-invasive potential was checked by Vimentin staining. Numerous irregular and dilated capillaries are visible in histology photos of all tumours V, VI and VIII.\ except for Δ133p53 siRNA tumours VI.\.

Figure 25. Δ133P53 knockdown prevents tumour growth and angiogenesis in CAM.



C.\ Angiogenic appearance presented as the percentage of absent – no visible tumour, vascular or avascular tumours following treatments with the different siRNAs – 10-18 tumours for each siRNA treatment. **D**.\ SNA-lectin staining allowed quantification of the capillary network invading the tumour by counting the number of green pixels on each field with Adobe Photoshop software. The whole surface of tumours has been counted because of capillary staining heterogeneity – at least 5 tumours / group. *, $P < 0.05$ vs the control siNON-treated group. Statistics done sith Student test.

Figure 25. Δ133P53 knockdown prevents tumour growth and angiogenesis in CAM.



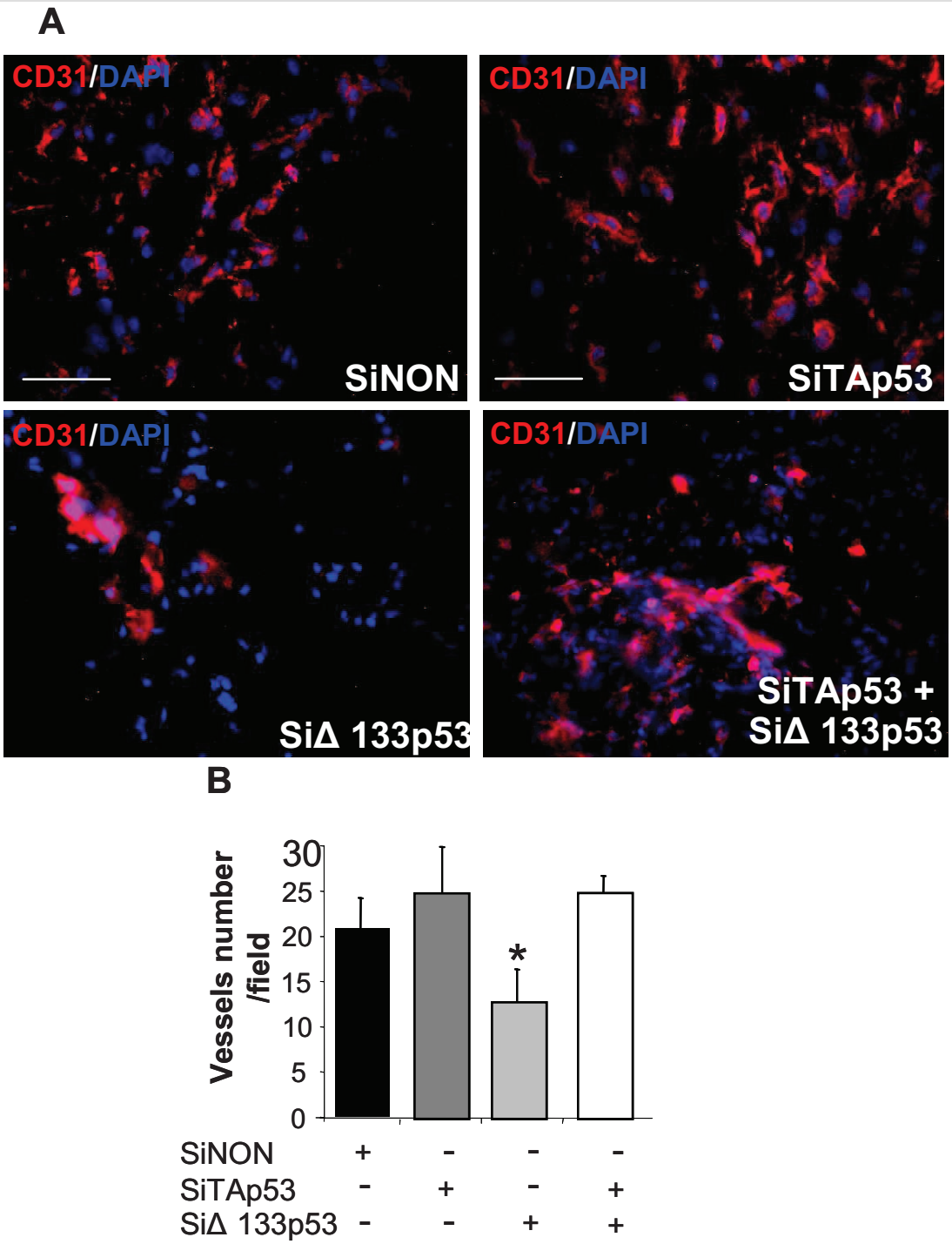
E.\ Tumour volume quantification – 11 to 16 tumours / condition. **F**.\ P53 protein expression in tumours analysed 4 days after implantation. Western blot is a merge of two different blots with 4 tumours for each condition. **G**.\ Quantification of p53 signal from Western blots from at least three tumours after normalization with β-actin. *, $P < 0.05$ vs the control siNON-treated group. Statistics done with Test Student. p53 immunostaining with Sapu antibody and β-Actin with β-Actin antibody

D. Δ133P53 knockdown prevents tumour angiogenesis and growth in mouse.

To study Δ133P53 involvement in tumour progression, siRNA-treated glioblastoma U87 cells were subcutaneously implanted in nude mice, and followed during 44 days – Figure 26. At day 7 after xenografts implantation, Δ133p53 knockdown resulted in significantly smaller tumours, an effect that was abolished in the double knockdown by SiTAp53 and SiΔ133p53 – Figure 26A. In addition, tumour sizes measured at day 44 confirmed the anti-tumoural effect of SiΔ133p53, whereas p53 knockdown had no significant effect. In contrast to the data obtained at day 7, the double knockdown did not abolish the anti-tumoural effect of SiΔ133p53 at day 44 – Figure 26B. Angiogenesis was quantified at day 7 by CD31 staining and showed that the SiΔ133p53-treated tumours were significantly less angiogenic at day 7 – Figure 26C and D.

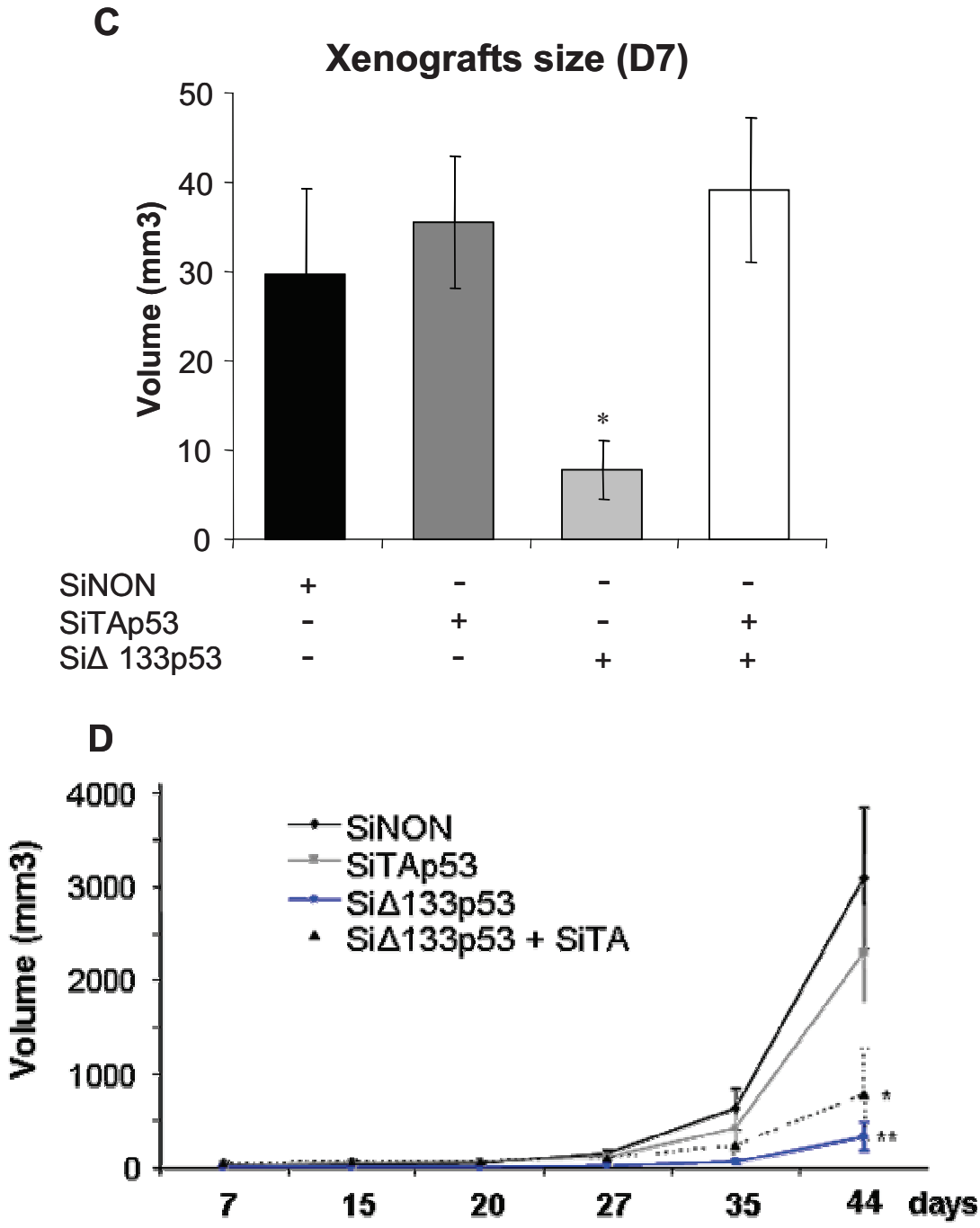
These data, fully consistent with that obtained in CAM, revealed that Δ133p53 might be a pro-angiogenic activator of tumour growth, contrasting with the well-known tumour suppressor feature of p53. They also suggested the existence of two mechanisms: a short term effect where Δ133p53 would antagonize p53 activity, and a long term activity on tumour progression resulting from Δ133p53 intrinsic activity.

Figure 26. Δ133P53 knockdown prevents U87 tumour growth and angiogenesis in mouse xenografts.



Knocked-down U87 were xenografted in nude mice. **A**.\ Tumour volumes were quantified at day 7 – 8 tumours / condition. **B**.\ Quantification of number of blood vessels/field. Asterisk indicates statistical significance. *, $P < 0.05$ vs the SiNON-treated group.Statistics done with Student test.

Figure 26. Δ133P53 knockdown prevents U87 tumour growth and angiogenesis in mouse xenografts.



Knocked-down U87 were xenografted in nude mice. **C**.\ Immunostaining to detect blood vessels in glioblastoma. Cryosections of murine xerographs of U87 cells were immunostained using anti-CD31 antibody. Nuclei were stained with DAPI – blue. Magnification x200. **D**.\ Evolution of tumour sizes until day 44. * = $p<0,05$ vs the SiNON-treated group.Statistics done with Student test.

E. **Δ133p53 modulates the angiogenic balance towards angiogenesis.**

The mechanism responsible for the pro-angiogenic activity of Δ133p53 was addressed by Taqman Low Density Array – TLDA – analysis of gene expression by siRNA-treated U87 cells. We used a Taqman human angiogenesis array providing a panel of 96 angiogenesis-related genes. In the control, expression of 14/39 anti-angiogenic genes – 35% – and 20/28 pro-angiogenic genes – 71% – was detected, confirming the strong angiogenic basal state of U87 glioblastoma – Figure 27A.

As regards anti-angiogenic factors, Δ133p53 knockdown resulted in induction of interleukin 12A – IL12A – and matrix metallopeptidase 2 – MMP2 – and repression of COL4A2 collagen – Figure 27B and Suppl data, Table I. MMP2 induction, also observed with p53 knockdown, was not specific of Δ133p53. In contrast, IL12A was repressed by p53 knockdown, but remained induced in the double knockdown, suggesting that its regulation by Δ133p53 is not dependent of p53.

Several pro-angiogenic factors were down-regulated by Δ133p53 knockdown, a profile drastically different from that obtained with p53 knockdown – Figure 27C – Angiogenin – ANG, Midkine – MDK – and hepatocyte growth factor – HGF. Interestingly, the main angiogenic growth factor VEGF is downregulated almost two times folowing Δ133p53 knockdown, which is a strong variation for such important facotr. This suggests that Δ133p53 could play a role in the regulation of VEGF levels. However, to prove that VEGF levels is deeply modified, further studies should be undertaken. On the contrary, other main proangiogenic factors like FGF1 and FGF2, remained unaffected. Regulation of MDK and HGF was abolished by the double knockdown, indicating a p53-dependent activity of Δ133p53. In contrast, ANG regulation was maintained in the double knockdown and thus probably corresponds to an intrinsic activity of Δ133p53. Δ133p53 knockdown also downregulated the angiogenic marker Angiopoietin-like 4 – ANGPTL4 – a regulation that is only partially removed by the double knockdown – Figure 27D.

The present study thus reveals that Δ133p53 is of first importance in the angiogenic balance and could be responsible for a strong angiogenesis stimulation that mainly contributes to acceleration of human glioblastoma progression. These data are novel and strongly contrast with the previously described anti-angiogenic activities of p53. Interestingly, Δ133p53 modulates the angiogenic balance differently of p53,

without affecting the classical angiogenic VEGF or FGF-dependent pathways, through regulation of a distinct signalling pathway involving other angiogenic factors including ANG, HGF and ANGPTL4. Data from the double knockdown indicate that Δ133p53 mechanism of action is partly dependent of p53, but also results from intrinsic activities.

Δ133p53 has first been shown to impair p53-mediated apoptosis by a dominant-negative effect (Bourdon *et al.* 2005). This is the most logical mechanism for an isoform lacking the TAD but still capable to tetramerize with p53. However, further studies of the zebrafish ortholog of Δ133p53, named Δ113p53, have shown that this isoform antagonizes p53-induced apoptosis via activating bcl2L, and is able to differentially modulate p53 target gene expression (Chen *et al.* 2009). As regards the ability of Δ133p53 to prevent cellular senescence, this activity seems to be mainly attributable to its dominant-negative effect over p53-induction of microRNA miR-34a (Fujita *et al.* 2009). In the present study, we clearly show that the regulation of several anti- and pro-angiogenic factors – mainly IL12A and ANG – observed following Δ133p53 knockdown is not dependent on the presence of p53, thus cannot result from a dominant-negative effect. Furthermore the effect of Δ133p53 on long term tumour progression in mouse is not dependent on p53. Among possible mechanisms, it can be proposed that Δ133p53 would interact with other transcription factors, such as HIF1-α. It might also exhibit a transactivating activity, as shown for ΔNp63, its corresponding isoform in the p53-related p63 gene: ΔNp63 contains a transactivation domain TAD2 and is able to transactivate the hsp-70 promoter (Ghioni *et al.* 2002; Wu *et al.* 2003).

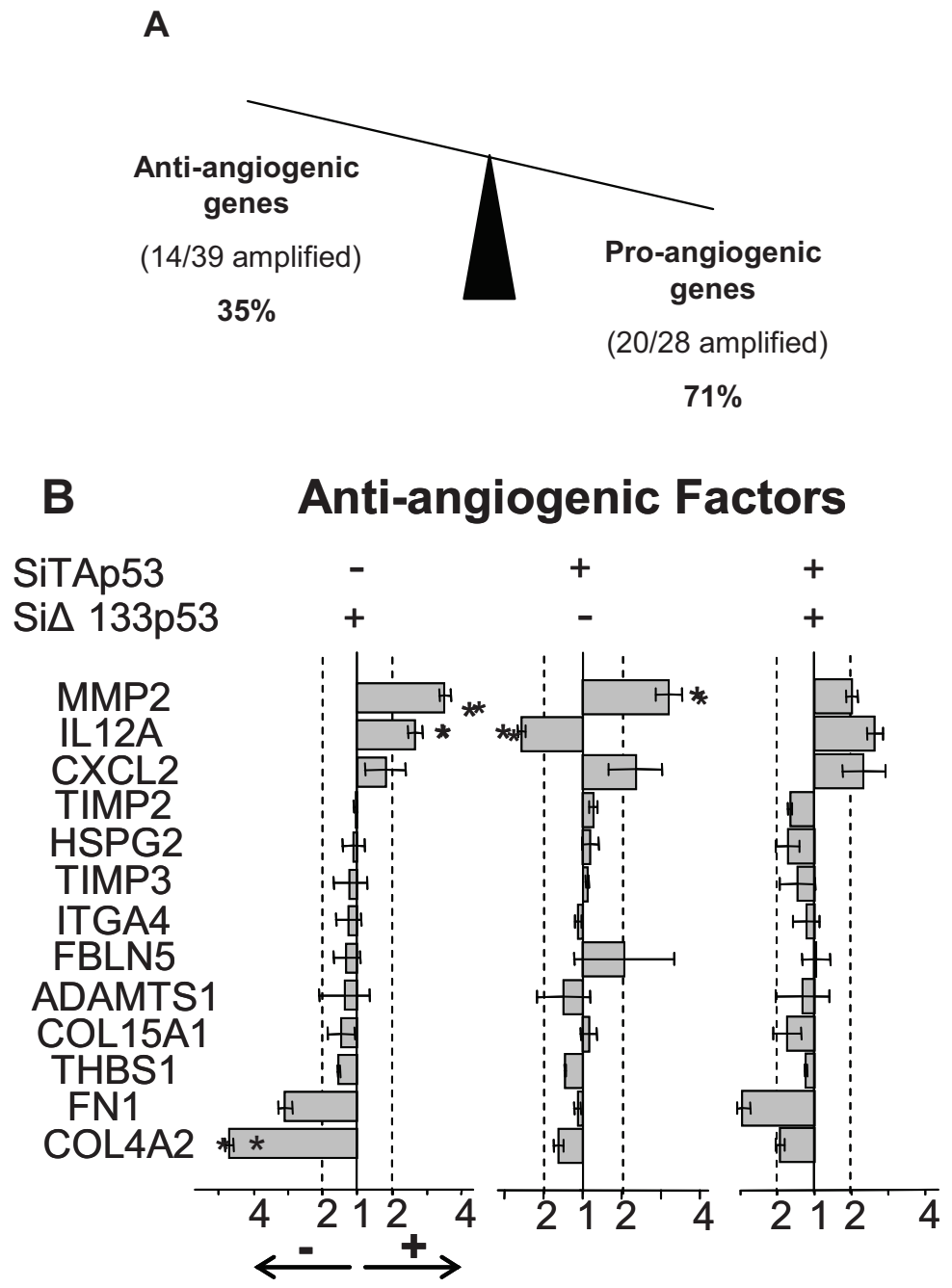
Our study shows that Δ133p53 knockdown results in p53 induction. This phenomenon takes place at both protein and mRNA levels, suggesting that Δ133p53 is able to repress p53 gene expression. Recent reports have shown that p53 transactivates the alternative promoter giving rise to Δ133p53, thus providing a negative feedback regulation of the p53 response, also shown for the p53 related gene p73 (Nakagawa *et al.* 2002; Chen *et al.* 2009; Marcel *et al.* 2010). Our data showing that Δ133p53 would be an inhibitor of p53 mRNA expression provide additional complexity to this feedback mechanism. In addition, they also provide a hypothesis of mechanism allowing Δ133p53 to antagonize p53 activity, different from its dominant-negative function.

A protumourigenic role of Δ133p53, contrasting with the tumour suppressor effect of p53, has been suggested by previous studies. In addition to its anti-apoptotic and senescence-inhibiting activities, Δ133p53 abnormal expression has been detected in

80% of mammary tumours from patients, whereas it is not expressed in normal breast tissue (Bourdon *et al.* 2005; Chen *et al.* 2009; Fujita *et al.* 2009). The present study demonstrates for the first time the direct role of Δ133p53 in acceleration of tumour progression. This role results, at least in part, from Δ133p53 capability to stimulate tumour angiogenic conversion. Such a feature may be critical in tumours exhibiting wild type p53, representing about 72% of grade I and 35% of grade II glioblastoma, for which angiogenesis has been recognized as a key event in their progression to malignancy (Ohgaki and Kleihues 2007; Wong *et al.* 2009).

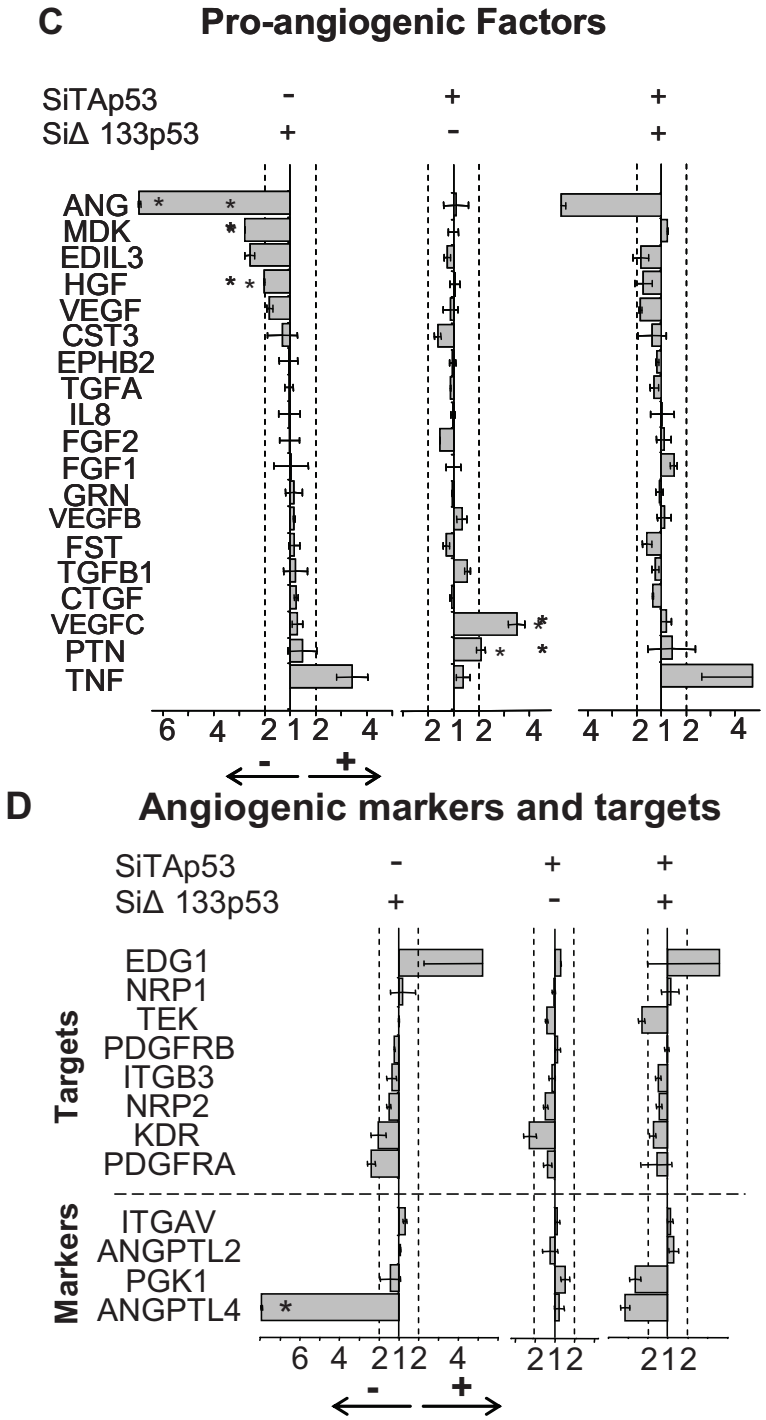
Our data may find an important relevance in anti-angiogenic therapeutics of cancer, presently based on angiogenesis inhibitors targeting the VEGF signalling pathway. Such treatments have been have proven to be efficacious in patient survival, associated with a chemotherapy. However, while antitumoural effects and survival benefit are often evident, relapse to progressive tumour growth has been recently reported, reflecting multiple mechanisms of adaptation to antiangiogenic therapies (Ebos *et al.* 2009; Paez-Ribes *et al.* 2009). In this context, Δ133p53 may have a critical role by allowing angiogenesis. Thus, targeting Δ133p53 may have a strong impact in cancer therapeutics.

Figure 26. Δ133P53 knockdown prevents U87 tumour growth and angiogenesis in mouse xenografts.



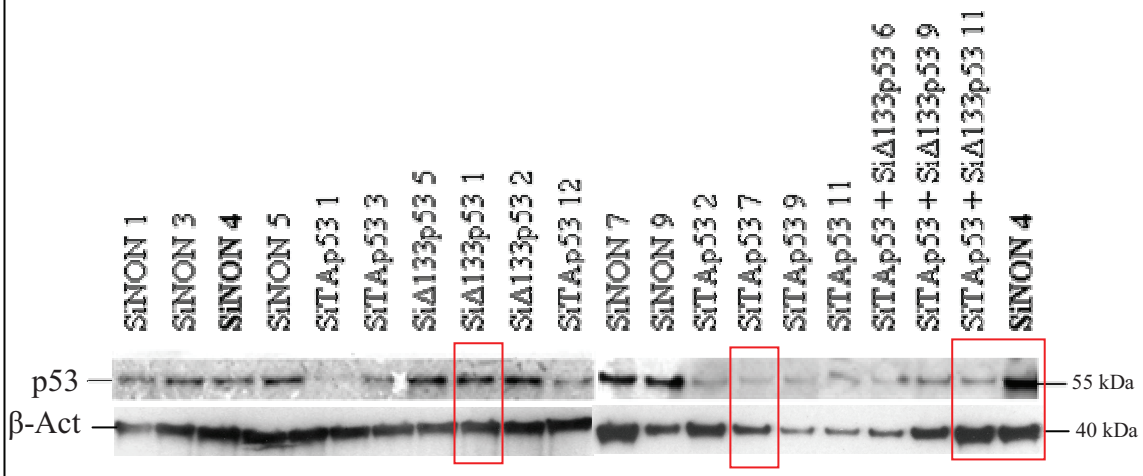
Expression of anti- and pro-angiogenic genes generated by Δ133p53 and p53 knockdown in U87 was analysed by TLDA. **A**.\ Expression of anti- and pro-angiogenic factors in U87 showing the number of genes amplified in basal conditions – SiNON. **B**.\, Histogram representing analysis of mRNA expression of anti-angiogenic genes. P=<0.05 vs the SiNON-treated group.Statistics done with Student test.

Figure 26. Δ133P53 knockdown prevents U87 tumour growth and angiogenesis in mouse xenografts.



C.\, Histogram representing analysis of mRNA expression of anti-angiogenic genes. pro-angiogenic **D**.\ markers and target genes – according to Applied Biosystem classification form. We considered as relevant repression (-) or induction (+) variations superior to two folds. Experiments were repeated twice. Details are presented in Sup. Table I.p=<0.05 vs the SiNON-treated group.Statistics done with Student test.

Supplementary Figure 1. p53 protein expression in U87 xenografts after 4 days (Extended western blot from Figure 25.F).



Western blot from tumor xenografts. Each condition is represented by several samples specifically numbered. Open red rectangles refer to the samples of the figure 25F.

Supplementary Table I: Angiogenesis Taqman Low Density Array of Δ133p53 and p53 knockdown.

	SiΔ133p53		SiTAp53		SiΔ133p53 + SiTAp53	
	Moy	p	Moy	p	Moy	p
Pro-Angiogenic factors						
TNF	**3.43**	0.08	**1.37**	0.20	**4.70**	0.16
ANGPT2	**2.15**	0.23	**6.94**	0.25	**-3.12**	0.13
PTN	**1.48**	0.28	**2.06**	**0.05**	**1.43**	0.37
VEGFC	**1.28**	0.21	**3.50**	**0.04**	**1.20**	0.27
CTGF	**1.23**	0.10	**-1.07**	0.28	**-1.35**	0.03
TGFB1	**1.21**	0.37	**1.55**	0.06	**-1.25**	0.20
FST	**1.16**	0.30	**-1.28**	0.17	**-1.60**	0.15
VEGFB	**1.15**	0.06	**1.33**	0.16	**1.11**	0.38
GRN	**1.15**	0.36	**-1.04**	0.08	**-1.09**	0.34
FGF1	**1.03**	0.48	**1.00**	0.50	**1.50**	0.08
FGF2	**-1.03**	0.48	**-1.53**	0.00	**-0.90**	0.36
IL8	**-1.03**	0.48	**-1.02**	0.43	**1.02**	0.48
TGFA	**-1.04**	0.43	**-1.12**	0.04	**-1.30**	0.21
EPHB2	**-1.07**	0.45	**-1.04**	0.39	**-1.17**	0.14
CST3	**-1.31**	0.38	**-1.62**	0.10	**-1.38**	0.36
VEGF	**-1.80**	0.09	**-1.12**	0.39	**-1.86**	0.04
HGF	**-2.01**	**0.01**	**1.06**	0.41	**-1.75**	0.22
EDIL3	**-2.58**	0.10	**-1.25**	0.18	**-1.84**	0.20
MDK	**-2.77**	**0.01**	**-1.01**	0.49	**1.24**	0.01
ANG	**-6.91**	**0.02**	**1.10**	0.44	**-5.08**	0.07
Anti-angiogenic factors						
VASH1	**3.70**	0.21	**4.84**	0.24	**1.84**	0.13
MMP2	**3.51**	**0.02**	**3.21**	**0.05**	**2.02**	0.20
IL12A	**2.64**	**0.04**	**-2.55**	**0.05**	**2.63**	0.17
CXCL2	**1.78**	0.20	**2.35**	0.15	**2.34**	0.20
TIMP2	**-1.05**	0.27	**1.27**	0.13	**-1.65**	0.04
HSPG2	**-1.12**	0.40	**1.20**	0.26	**-1.72**	0.19
TIMP3	**-1.21**	0.34	**1.11**	0.12	**-1.45**	0.16
ITGA4	**-1.27**	0.37	**-1.12**	0.23	**-1.22**	0.37
FBLN5	**-1.32**	0.17	**2.07**	0.28	**1.05**	0.49
ADAMTS1	**-1.38**	0.13	**-1.48**	0.05	**-1.32**	0.30
COL15A1	**-1.46**	0.28	**1.16**	0.29	**-1.74**	0.14
THBS1	**-1.53**	0.03	**-1.44**	0.02	**-1.24**	0.25
FN1	**-3.08**	0.09	**-1.13**	0.21	**-2.94**	0.09
COL4A2	**-4.70**	**0.05**	**-1.61**	0.10	**-1.93**	0.06
Angiogenesis targets						
EDG1	**5.23**	0.15	**1.29**	0.38	**3.62**	0.25
NRP1	**1.19**	0.37	**-1.04**	0.32	**1.14**	0.36
TEK	**-1.01**	0.33	**-1.35**	0.04	**-2.32**	0.06
PDGFRB	**-1.22**	0.01	**1.15**	0.17	**-1.04**	0.34
ITGB3	**-1.36**	0.18	**-1.10**	0.26	**-1.48**	0.09
NRP2	**-1.51**	0.06	**-1.41**	0.08	**-1.42**	0.08
KDR	**-2.04**	0.15	**-2.15**	0.12	**-1.74**	0.07
PDGFRA	**-2.39**	0.08	**-1.32**	0.17	**-1.55**	0.31
Angiogenesis markers						
ITGAV	**1.30**	0.06	**1.13**	0.16	**1.12**	0.19
ANGPTL2	**1.07**	0.09	**-1.20**	0.31	**1.32**	0.14
PGK1	**-1.44**	0.28	**1.49**	0.09	**-2.65**	0.09
ANGPTL4	**-7.93**	**0.02**	**1.22**	0.20	**-3.15**	0.07

Supplemental Table II : Sequences of siRNAs and oligonucleotides.

SiΔ133p53-1	5’- GGAGGUGCUUACACAUG[dT][dT] -3’
SiΔ133p53-2	5’-CUUGUGCCCUGACUUUCAA[dT][dT]-3’
SiTAp53	5’-GGAAACUACUUCCUGAAAA[dT][dT]-3’

FLp53 Forward	5’ CAGCCAAGTCTGTGACTTGCA 3’
FLp53/Δ133p53 Reverse	5’ GTGTGGAATCAACCCACAGCT 3’
FLp53/Δ133p53 Probe	5’TCCCCTGCCCTCAACAAGATGTTTTGCC-3’
Δ133p53 Forward	5’ ACTCTGTCTCCTTCCTCTTCCTACAG 3’
18S Forward	5’ CAACTAAGAACGGCCATGCA 3’
18S Reverse	5’ AGCCTGCGGCTTAATTTGAC 3’

F. Further insights into Δ133p53 role in angiogenesis

F.I. Verification of SiΔ133p53-2 efficiency

In parallel to SiΔ133p53-1 siRNA, we undertake the verification of another siRNA specific to the Δ133p53 mRNA in U87 cell line, named SiΔ133p53-2. To verify siRNA efficiency, I did as well as in Figue 23 RNA quantification and included the previous SiΔ133p53-1 for comparison.– Figure 28. The results showed that besides the effects observed in Figure 23, the SiΔ133p53-2 was also capable to induce the Δ133p53 mRNA degradation but to a less extend – 40% compared to 60% for SiΔ133p53-1. These differences are explained by either the sequence stringency that triggers RNA interference and by the different factors that could be bound to the region of the Δ133p53 5'UTR mRNA. However SiΔ133p53-2 induced the overexpression of p53 mRNA in the same amplitude of SiΔ133p53-1. Interestingly the SiTAp53 did not induce inhibition of Δ133p53 mRNA expression. Indeed, as p53 is known to induce the activity of Δ133p53 promoter, we would have expected decreased expression of Δ133p53mRNA. This dichotomy of response between knockdown and overexpression of p53 may be explained by the fact that overexpressed p53 induce a huge set of genes that participate and modify p53 to activate its transcription activity. On the contrary when disrupted or at "normal" levels, p53 does not reach the threshold sufficient to induce genes. Also of interest, the double knockdown SiΔ133p53-1/SiTAp53 results in the expression of unchanged p53 mRNA levels and shows that the potency of p53 siRNA disruption is balanced by the indirect effects of Δ133p53 mRNA disruption.

I further analysed the SiΔ133p53-2 effects in functional analysis. I thus did CAM assays similarly to those presented in Figure 25. SiΔ133p53-2 tumours showed a decrease of the number of vascularized tumours – Figure 29. These results show us that SiΔ133p53-2 is also potent to inhibit angiogenesis.

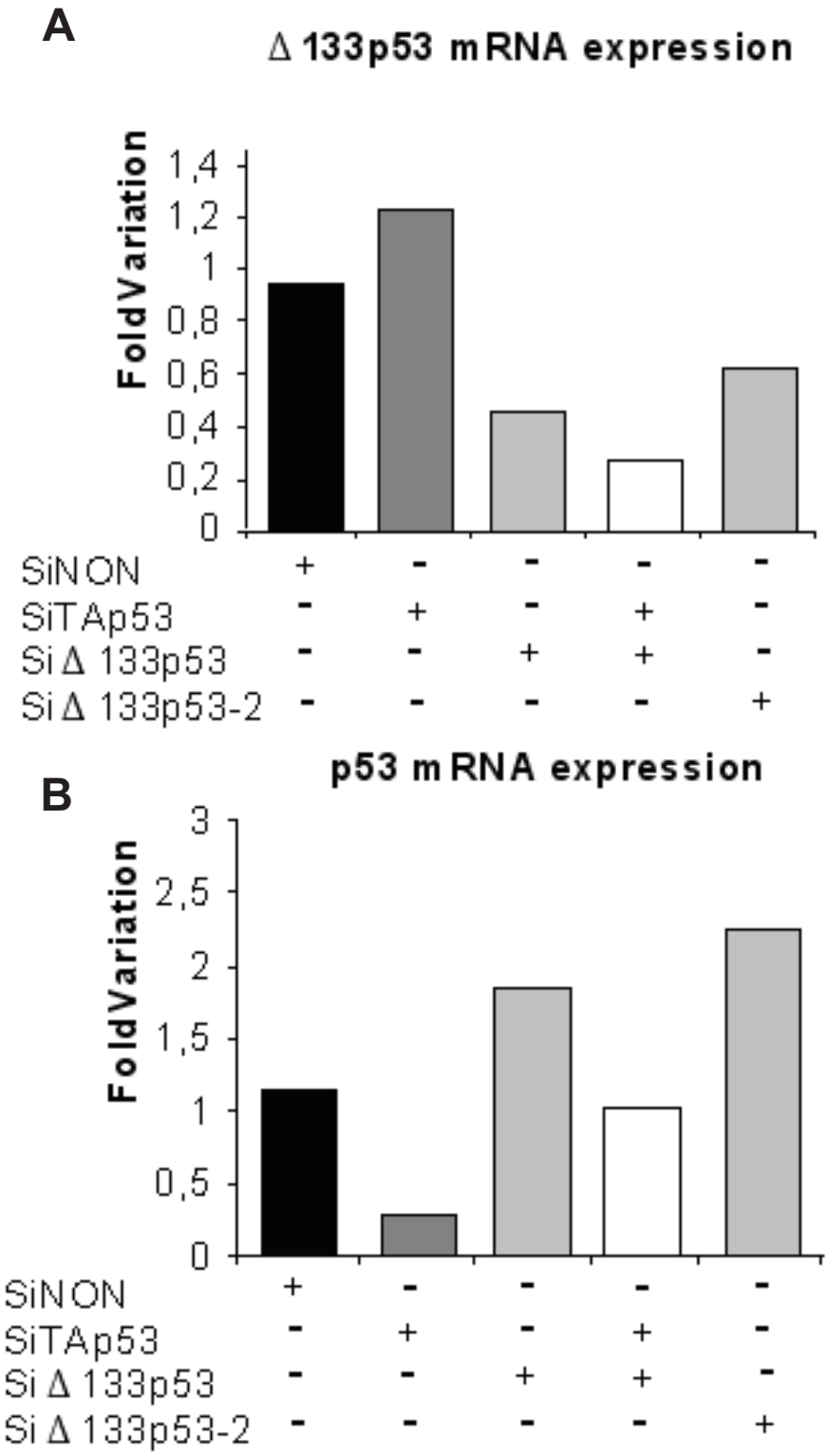


Figure 28. Δ133p53 A.\ and p53 B.\ mRNA assessment U87 cells following SiRNA treatment using QRT-PCR. For Δ133p53 mRNA quantification 100ng of cDNA were used after RT, for p53 mRNA 10 ng. Experiments were repeated twice in duplicate, Mean is represented. Sample quantity is normalized with 18S rRNA using the $2^{-\Delta\Delta Ct}$ method and set to percentage of control. Sequence of SiRNAs are in Suppl. Table II.

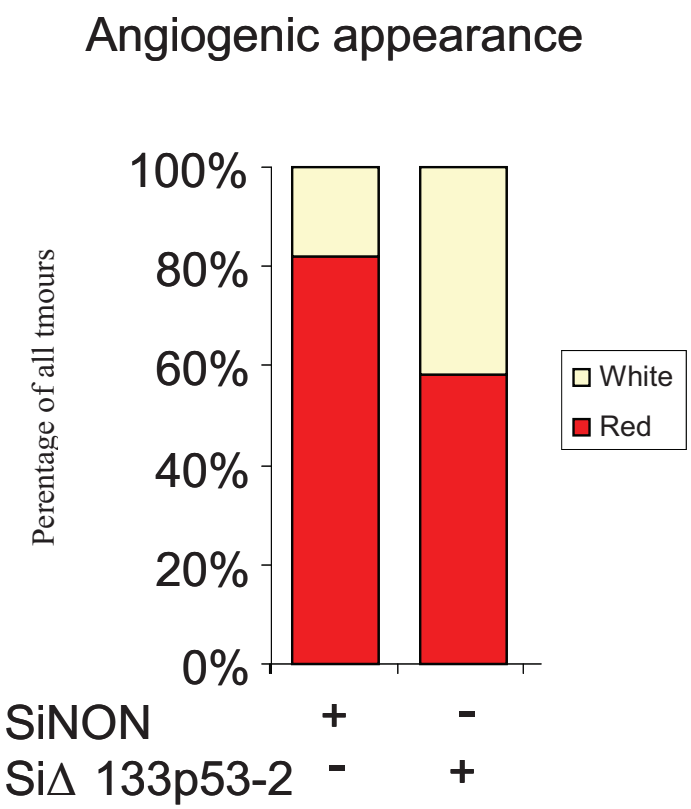


Figure 29. Angiogenic appearance presented as the percentage of vascular – red - or avascular - white - tumours following treatments with the different SiNON and SiΔ133p53-2 SiRNAs. 15 SiNON tumours were vascularized at 81% and 12 SiΔ133p53-2 vascularized at 58%. Experimental procedure was the same as in Figure 25.

F.II. EC migration & proliferation assays with U87 conditioned media.

F.II.1. EC treatment with conditioned media of siRNA transfected U87.

Before starting *in vivo* studies I did some *in vitro* preliminary work on endothelial cells using adult bovine aortic endothelial cells – ABAE – and HUVE cells – HUVEC. The results obtained with the HUVEC is already showed above in the paper but I first worked with ABAE cells, which are much more convenient to work. I used the same protocol as mentioned for the HUVEC to obtain the conditioned media. Data show that SiTAp53 treatment enhances cell migration, whereas SiΔ133p53 has an inhibitory effect – Figure 30A and Figure 30B. ABAE cell migration was also studied using Boyden chamber assays. The main difference between Boyden chamber and wound healing assays – apart from the technical aspect of the experiment – is that in Boyden chambers ECs actively cross a membrane and thus show an active migration. In wound healing migration can be mixed up with spreading of a layer of cell into the empty part of the Petri dish, and with proliferation if the experiment last long. The results in Boyden chamber confirmed our results in wound healing and exhibited the same pattern: inhibition of migration with SiΔ133p53 and increase of migration with SiTAp53 conditioned medium – Figure 31.

Similarly to HUVEC I did MTT experiments to analyse the proliferation of endothelial cells between the different conditions and obtained the same results: p53 and Δ133p53 knockdown did not have an effect on endothelial cell proliferation – Figure 32.

These results showed that conditioned medium of SiΔ133p53 treated U87 cells inhibits in a similar manner migration of both lines without affecting their proliferation. However, SiTAp53 treatment resulted in increased of ABAE migration, which was not visible in HUVEC. Such a difference may be explained first by the overall migration rates. As we can notice, migration was almost complete and the wound recovered by HUVEC, but the recovery was just beginning in the ABAE cells. Increased migration was hence easier to observe in ABAE cells. Another explanation relies on the differences between these two cell lines that could explain more responsiveness in ABAE cells.

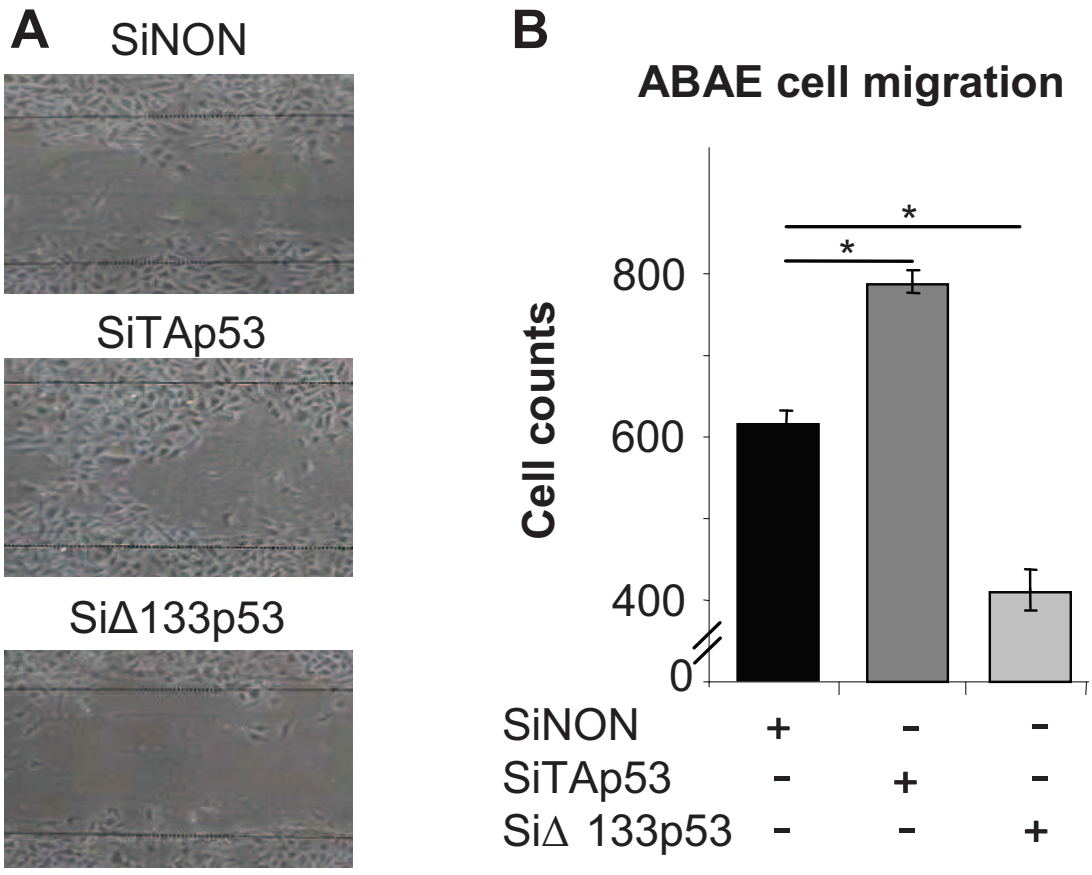


Figure 30. ABAE cell migration after treatment of U87 SiRNA treated condition medium. In summary, as in figure 24, U87 media were harvested 48h after SiRNA treatment and dead cells and cell framents removed by centrifugation. A wound is done with the tip of a 10 µl cone and conditioned media introduced for 18h. A.\ Photos are taken at the beginning and the end of the experiment. Experiment done twice, Mean is represented. B.\ Cells counts correspond to the number of cells that have invaded the wound. P<0,05 vs SiNON-treated Control. Statistics done with Test Student.

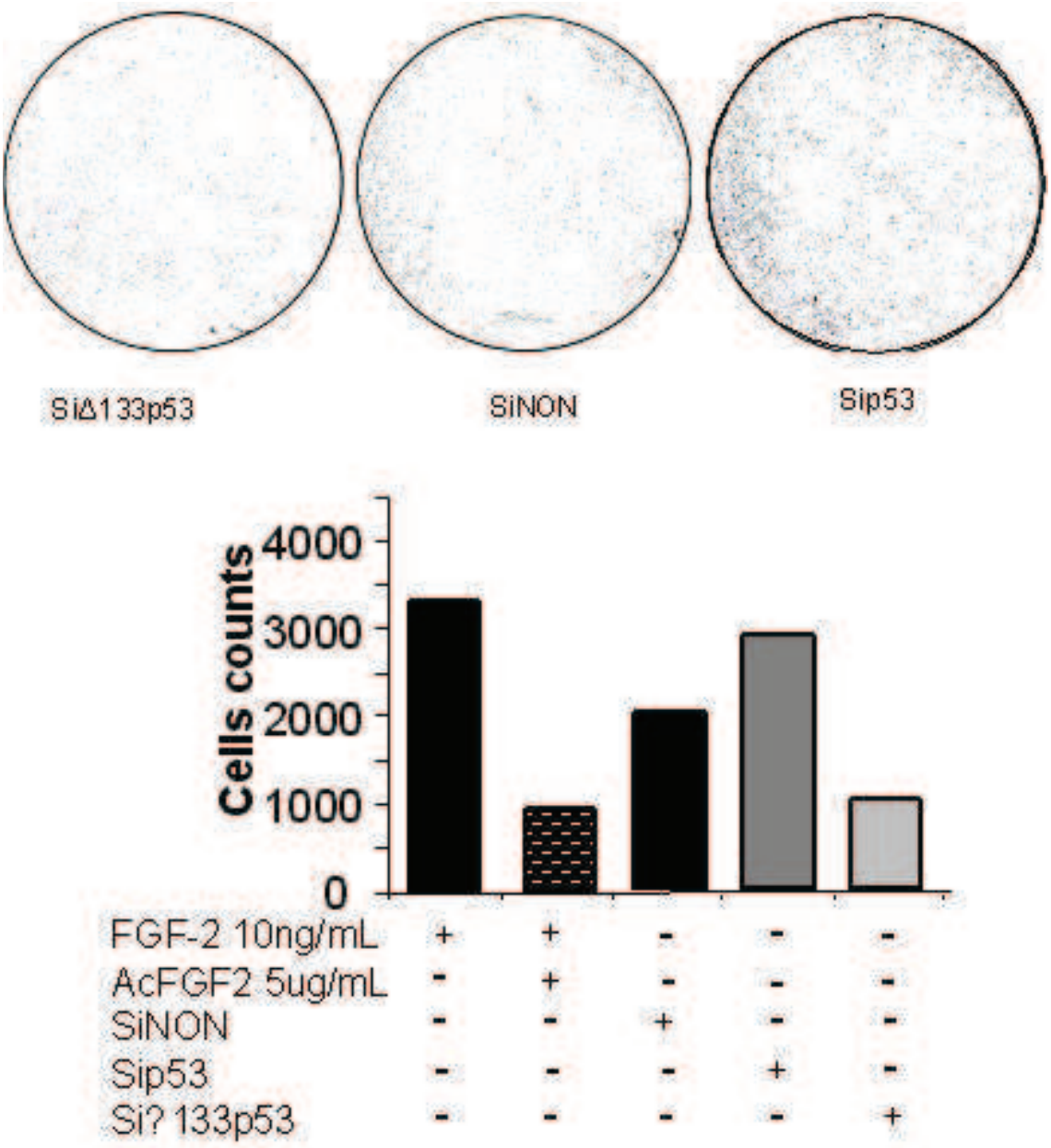


Figure 31. Boyden Chamber assay. U87 cells transfected with the above mentioned SiRNAs are cultivated for two days in the lower compartment. As control, media containing FGF-2 or a FGF-2 blocking antibody were used. In the upper compartment, 100000 ABAE cells were deposited in the insert – for a 24 well/plate boyden chamber - and filled with condition media from the lower compartment for six hours. Experiment done twice, Mean is represented. At the end of the experiment insert were incubated in paraformaldehyde (4 % p/v) and stained with trypan blue, and cleaned to allow the counting of the migrating cells. Experiment done twice. Cell counts were done with the freeware ImageJ.

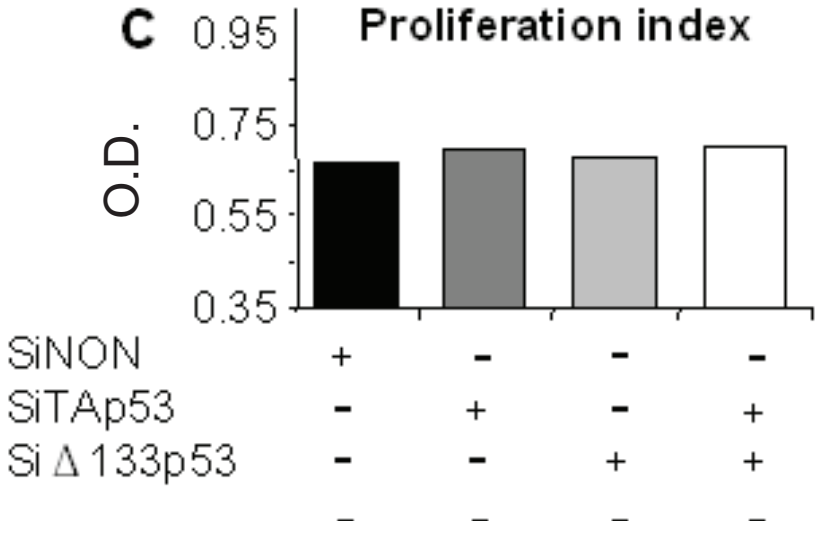


Figure 32. MTT experiment of ABAE cells after treatment of U87 SiRNA treated condition medium. Experiment done twice, mean is represented, and measured after 48h of conditioned medium treatment. Experiment protocol was the same as Figure 24A-B.\

F.II.2. Conditioned media from U87 cell line overexpressing p53 isoforms.

Finally we performed experiments using conditioned media from U87 transfected cells with plasmids expressing p53 isoforms constitutively – one isoform per condition. Overexpression of p53 was controlled by Western blot and quantitative RT PCR – Figure 33 and 34. The relative quantification of p53 mRNA isoforms reflects the fold increase of each overexpressed isoform compared to the endogenous one. As I put the same amount of each plasmid for each transfection – 1ug/6 cm dish of U87 cell line – the differences of fold variation reflect only the difference of endogenous expression between full length p53 and short length isoforms. Indeed, using a common set of primer for all isoforms I already noticed that full length p53 mRNA is expressed between twelve to sixteen times more than the Δ133p53 isoforms. I then did a western blot to control the expression of the p53 isoforms. The result I obtained can appear quite surprising, but they can be explained by at least two main reasons. First the antibody I used for p53 detection – Sapu – reveals with more efficiency full length isoforms than Δ133p53 ones and more Δ133p53α isoform than Δ133p53β and Δ133p53γ isoforms. The following table show a set of antibody that is able to bind specifically p53 isoforms – Table 5. However we still do not have specific antibodies for the γ splice isoforms. We noticed that the γ spliced isoforms are much more difficult to reveal since they seems to be highly unstable.

Wound healing assays were however performed after EC treatment with conditioned media of U87 transfected by plasmids expressing the different isoforms. We first observed that p53α overexpression, in a consistent manner with the literature, inhibits ABAE cell migration. Secondly, we found that overexpression of p53β induced a milder inhibition of ABAE cell migration. This means that p53β is also able to block EC migration and modulates the secretion of tumours. For this, p53β might have also an impact on microenvironment surrounding the tumour. On the contrary we did not find significant modification of ABAE migration with p53γ overexpressed conditions. Concerning the Δ133p53α, Δ133p53β and Δ133p53γ isoforms we found respectively inhibition for α and β isoforms and enhancement for γ isoform.

Table 5. Specific human p53 antibodies. from Bourdon et al. 2007

	Mouse monoclonal				Rabbit polyclonal		Sheep Polyclonal
Epitope (aa)	DO-1/DO-7 (20–25)	1801 (46–55)	DO-12 (256–270)	421 (372–382)	KJC8 (β)	CM1 recomb p53	Sapu
p53	++	++	+	+	–	+++	+++
p53β	++	++	+	–	++	++	++
p53γ	++	++	+	–	–	++	++
Δ40p53	–	++	+	–	–	++	++
Δ40p53β	–	++	+	–	++	++	++
Δ40p53γ	–	++	+	–	–	++	++
Δ133p53	–	–	+	–	–	+	++
Δ133p53β	–	–	++	–	++	+	+
Δ133p53γ	–	–	++	–	–	+	+

These results seem in contradiction with data obtained from the knockdown experiments where SiΔ133p53 clearly inhibits EC migration. Although we must remember that SiΔ133p53 blocks expression of the three Δ133p53 isoforms – α, β, and γ – it is unlikely that Δ133p53γ would be responsible on the effect on migration as the endogenous isoform is not detected in U87, even by qRT PCR – Figure 23. Our hypothesis is that the ratio of Δ133p53 versus p53 is of first importance for endothelial cell migration and possibly other related effects. When present in a low stoechiometry to p53, Δ133p53 would have its specific effect leading to silencing of p53 expression and expression of angiogenic factors as shown above with the Δ133p53 knockdown – Figure 27. However when expressed in non physiological conditions by plasmid transfection, Δ133p53 – α and β – seem able to have the same effect of p53 on migration, maybe by forming tetramers with p53 and having in that case a stimulatory effect rather than a dominant negative effect. Thus it would be important to tightly control the level of expression of the isoforms in order to clearly decipher the underlying mechanisms.

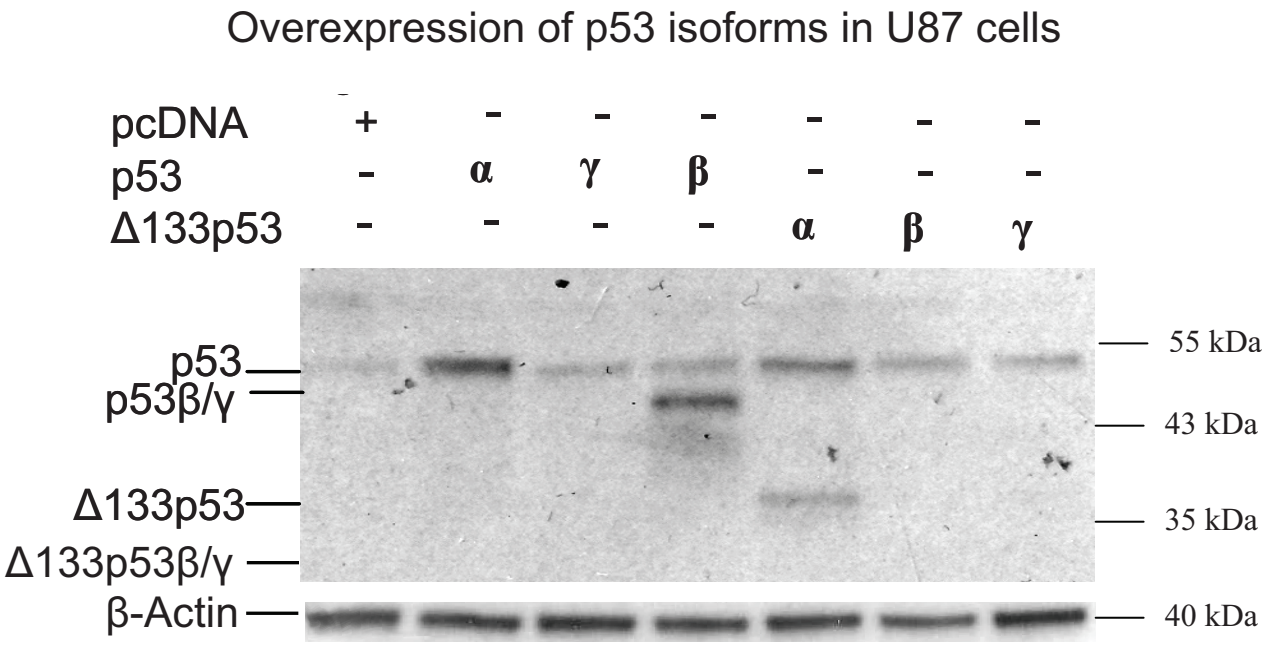


Figure 33. Western blot showing p53 isoforms protein expression following their overexpression by plasmid transfection in U87. Same amount of plasmid was used for each isform – 1µg per plasmid per 6 well/plate following indications. p53 immunostaining with Sapu antibody and β-Actin with β-Actin antibody

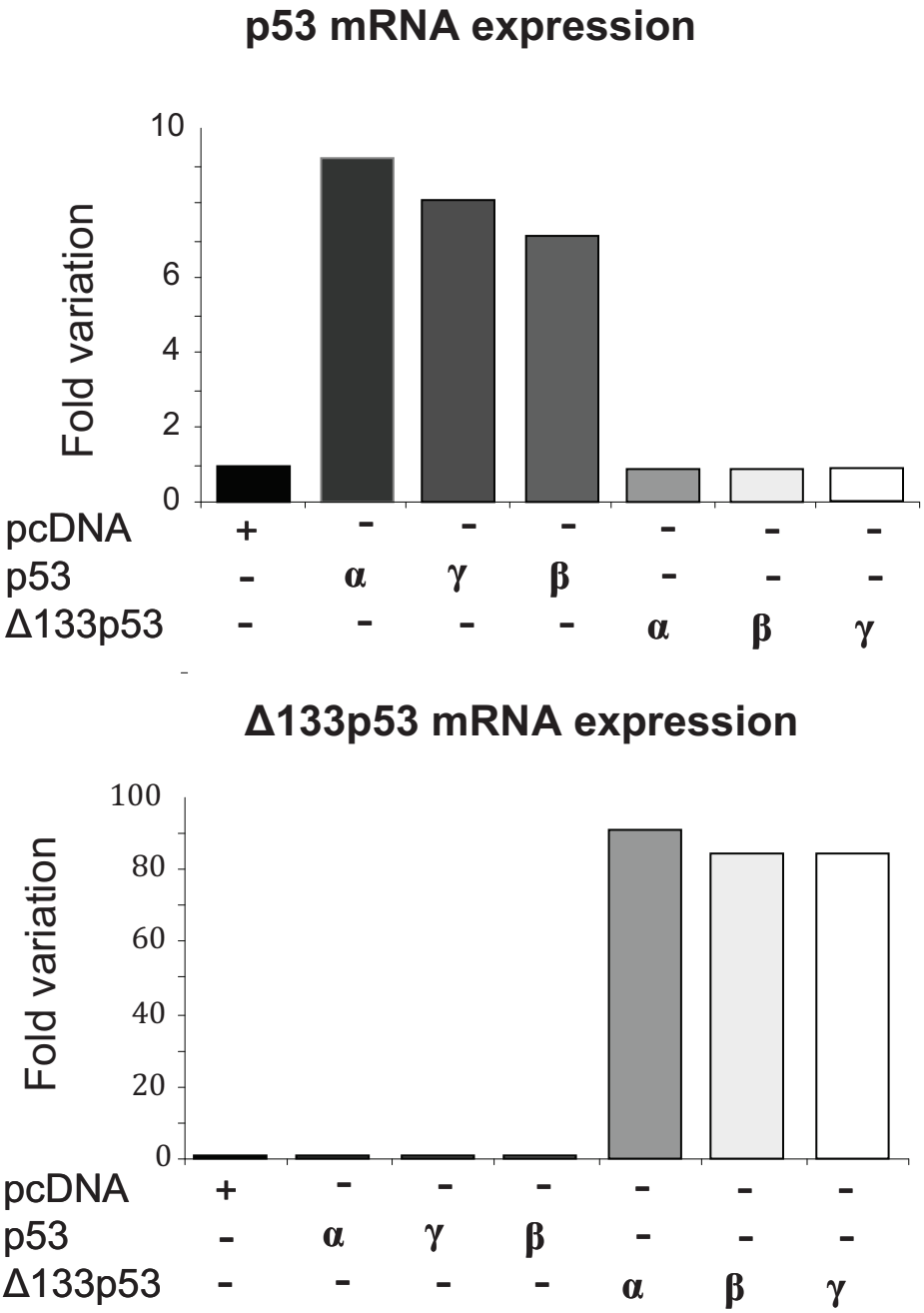


Figure 34. p53 A.\ and Δ133p53 B.\ mRNAs relative expression following siRNA treatment – qRT-PCR. Experiments were repeated twice in duplicate. Sample quantity is normalized with 18S rRNA using the $2^{-\Delta\Delta Ct}$ method and set to percentage of control. Experimental protocol described in figure 33 and qRT-PCR done according to the material and methods.

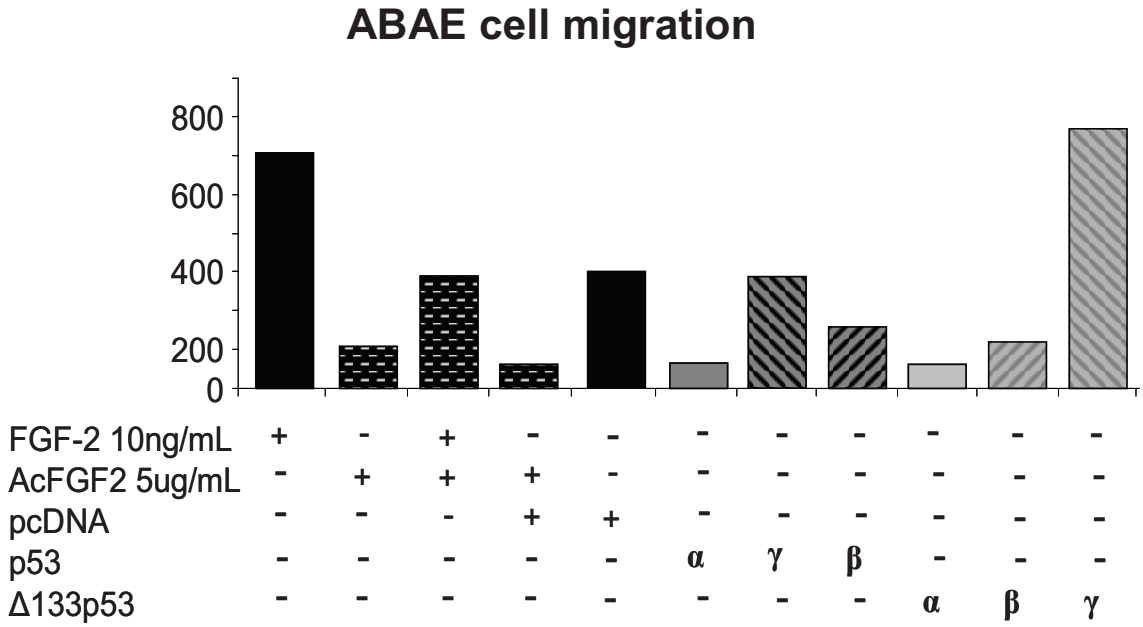


Figure 35. Quantification of wound healing assay of ABAE cells treated with conditioned media from overexpressing p53 isoforms in U87 cells. Experimental protocol was the same as Figure 24.

F.III. Construction and verification of inducible Δ133p53 isoforms lentivectors

In order to be able to express controlled levels of Δ133p53 isoforms, we constructed a set of inducible lentivectors overexpresssing p53 isoforms. Transduction of lentivectors has couples of interests in research and in our particular case. It is possible to transduce all types of cells and get instantly a population of cells expressing at 100 % the gene of its interest. Lentivectors, as a subfamily of retrovirus family is able to integrate the genome and be expressed for long periods. Furthermore, inducible vectors allow controlling the synthesis of the protein of interest. Presented in the figure 36, here are the specific features of the 3rd generation lentivector pTRIP-TRE tight GFP vector, provided by the BiVIC platform in Toulouse. Of importance are the 5' and 3' long terminal repeats – LTR – modified for better safety and the pTRE promoter construction containing a minimal CMV promoter fused to a Tetracyclin Responsive Element.

We first cloned the Δ133p53α, β and γ isoforms – from pCMV plasmids containing each isoform – into the multiple cloning site – MCS – of the pTRIP-TRE tight MCS vector by a tri-molecular ligation. The control the contruct inducibility, we transfected these plasmids with/without a pTet-off plasmid – allowing constitutive expression of the isoforms in the absence of doxycyclin – into U87 cells and assessed the level of transfection by FACSCAN and measured pTet-off induction by western blot. As a control we transfected as well the pcDNA plasmids containing each isoform.

FACSCAN experiments showed us a level of transfection of 15% of cells with either a pcDNA-GFP plasmid or a pTRIP-TRE tight GFP vector. This shows us that despites previous experiments with better rate – more than 80% – transfection is a sensitive a variable technique that would strongly change results of an experiment.

Protein expression of the transfected inducible plasmids is shown in the figure 37 and revealed in Western by Sapu antibody. As expected, pTRIP-Δ133p53 isoforms and pTetOff co-transfections induced strong expression of the Δ133p53 isoforms whereas Δ133p53 isoforms expressed control plasmid did not yield sufficient expression to observe it in western. Strikingly, pcDNA-based as well as pTRIP-based plasmids containging the isoforms did not express the same amount of isoforms,

probably due to their different stabilities. Furthermore, as explained in the B.II.2 paragraph, Δ133p53α, β and γ isoforms are not equally sensitive to Sapu revelation.

Presently, lentivectors are being produced to be able to analyse the effect of expression of each isoform on angiogenesis and tumour growth.

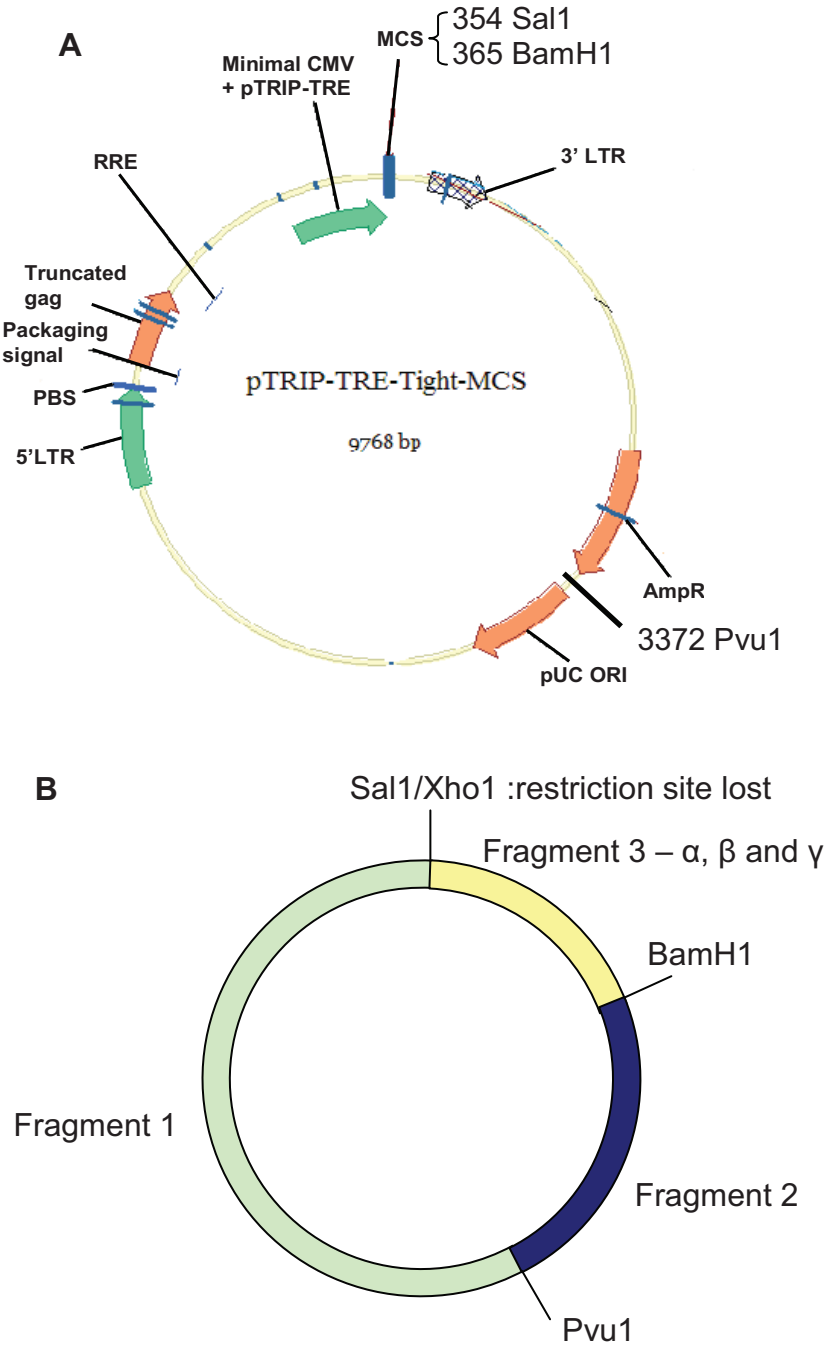


Figure 36. A.\Schema of the pTRE-TRIP-Tight-MCS used for the cloning of the different Δ133p53 isoforms.
MCS: Multiple cloing site; 3' and 5' LTR: Long terminal repeat - modified; AmpR: Amicilin Resistance; pUC ORI: pUC replication origin; RRE: Rev responsive element; minimal CMV + pTRIP-TRE: tetracycline responsive element fusioned to minimal cytomegalovirus promoter.
B.\ Strategy for the construction of the pTRIP-TRE- Δ133p53 plasmids: tri-molecular ligation of Fragment 1 from Sal1/Pvu1 digestion of pTRE-TRIP plasmid, Fragment 2 from BamH1/Pvu1 digestion of pTRE-TRIP plasmid and Fragment 3 – α, β, γ - from Xho1/Bamh1 digestion of the pCMVΔ133p53α, β and γ.

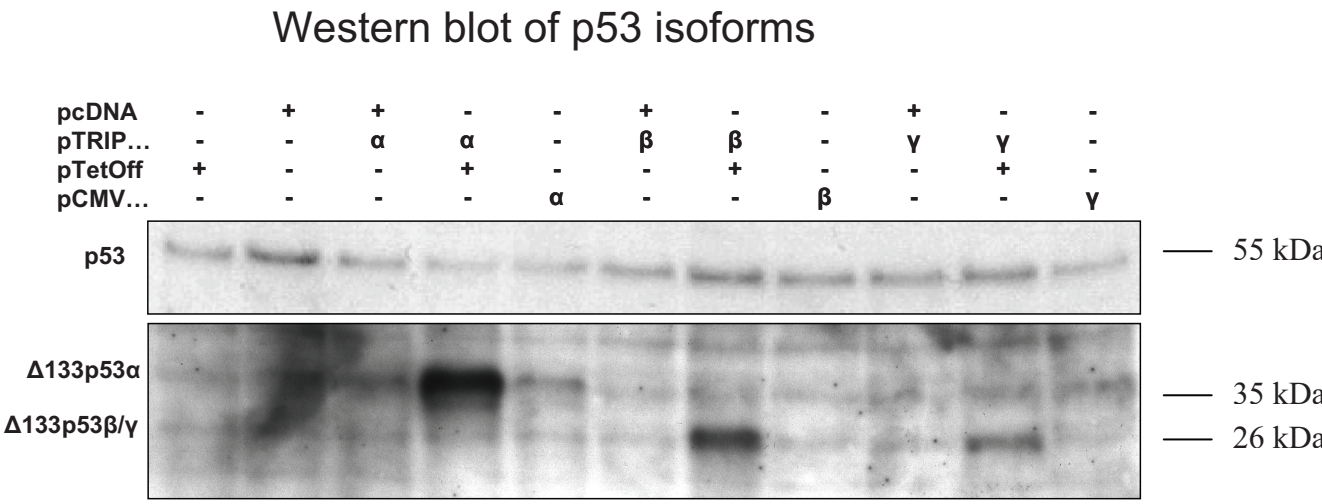


Figure 37. Western blot of p53 and the Δ133p53 isoforms in U87 cells following overexpression by plasmid transfection. p53 and Δ133p53 immunostaining with sapu antibody with different exposures.

F.IV. Analysis of the secreted angiogenic factors from U87 cells.

All the effects observed in ECs migration and tubulogenesis are due to the composition of the conditioned media. In order to analyse them, I first performed an ELISA –Fig 38. After 48h of treatment, I harvested the media and evaluated the levels of FGF-2 protein in the supernatants. As we can see no remarkable differences of FGF2 secretion, this argued against an involvement of FGF2 in the effect de Δ133p53 on angiogenesis.

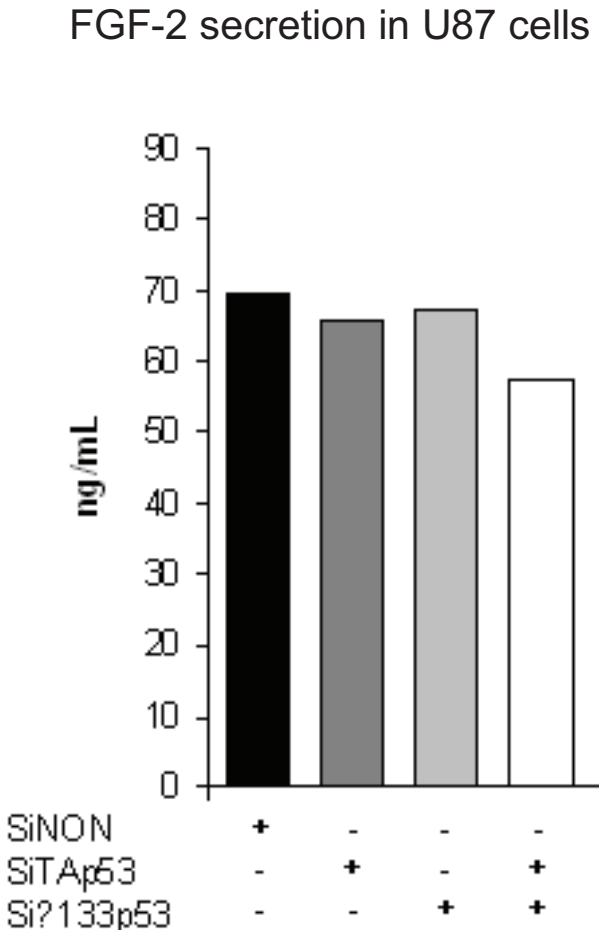


Figure 38. FGF-2 ELISA assay of conditioned media from U87 cells treated by SiRNAs. Experimental protocol for harvesting the conditioned media has been described in Figure 30. Procedures for ELISA were done following manufacturer's protocol. Experiment repeated twice in duplicate and Mean is represented

F.V. Proliferation of SiRNA transfected U87 cell lines

As the effects of p53 and Δ133p53 are intrinsically related with cell survival, proliferation and death, we next analysed the proliferation of siRNA transfected U87 cells by MTT. The results are presented in the following figure 39. This shows us first that there is barely any difference in proliferation between control treated cells and the p53 knockdown cells. On the contrary we can observe that SiΔ133p53 treatment strongly affects U87 proliferation: At day 1 there is less catabolism of MTT products revealing less proliferating cells. When looking at MTT catabolism we can also see that the proliferation rate is smaller in Δ133p53 knockdown cells. When SiTAp53 is jointly added to SiΔ133p53 treatment there is no notable difference. This means that the effect observed here in proliferation in not dependent of p53. Endogenous Δ133p53 is then able to induce cell proliferation independently of the full length p53 isoforms. However in these cancerous cells, SiΔ133p53 treatment does not completely inhibit proliferation and still rapidly become confluent *in vitro* – from my own experience. Anyway these results could explain why in mice the SiΔ133p53 treated U87 tumours progress not as quickly as the control and the SiTAp53 ones. Additionally these results confirm the taqman low density array, showing specific targets genes for Δ133p53.

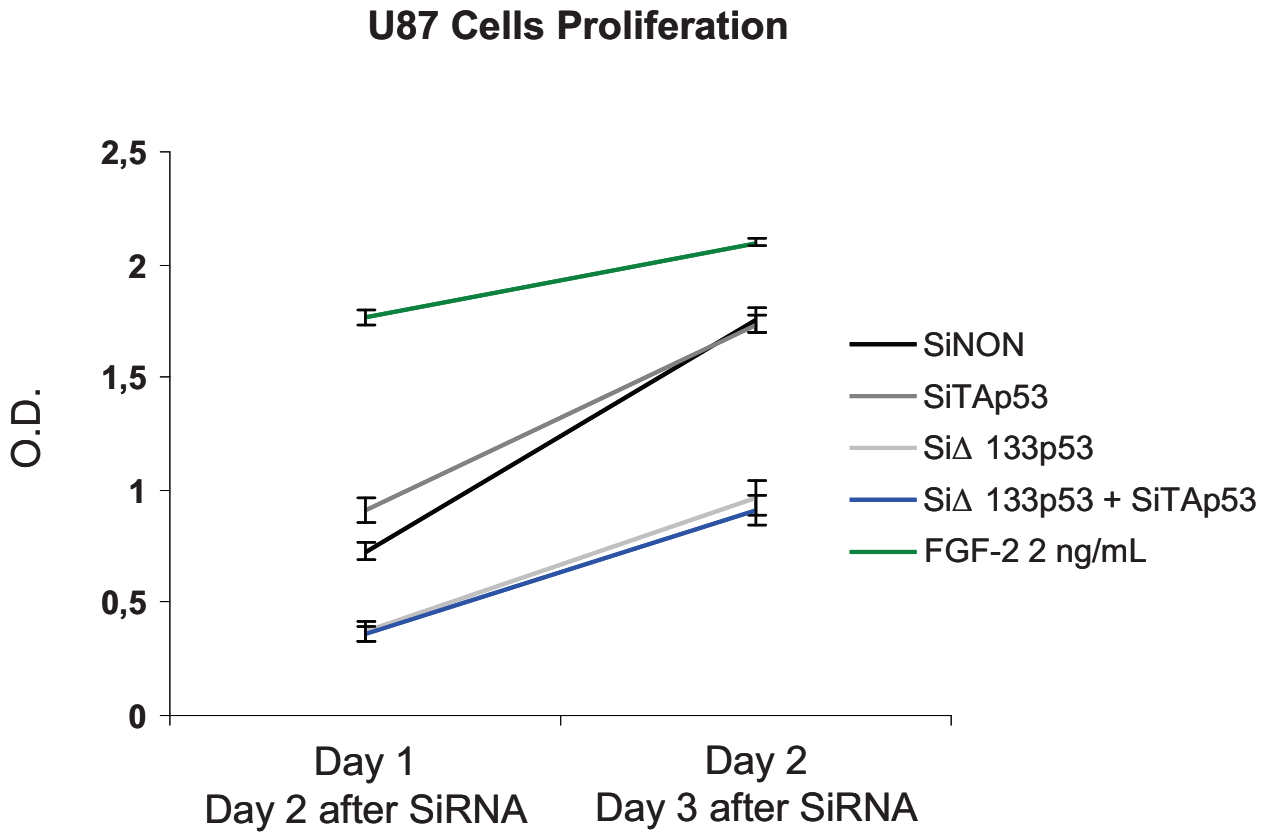


Figure 39. MTT proliferation assay in U87 cells treated by SiRNAs or FGF-2. Exprimental protocol is the same as Figure 24.

F.VI. Angiogenesis & tumour proliferation assessment in mice xenografts

In order to link the differences in tumour progression in mice xenografts with common parameters of tumour growth, observed when measuring the evolution of the tumour size until day 44, I analysed the tumours for their proliferation index and their vascularity. I used in immunofluorescence the Ki-67 proliferating cells marker, the CD31 endothelial marker and DAPI as nucleus marker. This allowed me to measure the proportion of Ki-67 positive cells and the total endothelial cell staining in tumour cells. Results are shown in the figure 40. As we can see none of the siRNA transfected tumours induced a difference in both parameters. This is due to the fact that tumours have been harvested too late to observe any phenotype. At this time the effects of siRNA are no more visible. However it seems that siRNAs can be effective for long time when transfected and then implanted into nude mice. For instance Filleur *et al.* found that their siRNA targeting VEGF-A knocked down its target at 50% until day 13 and was correlated with strong inhibition of cJ4 rat fibrosarcomas xenograft growth (Filleur *et al.* 2003). At day 44, it seems quite normal, although I have not done the proper measurements, that siRNA have not effect.This is the reason why we decided to do the experiment again and sacrifice the mice at day 10 – Figure 26.

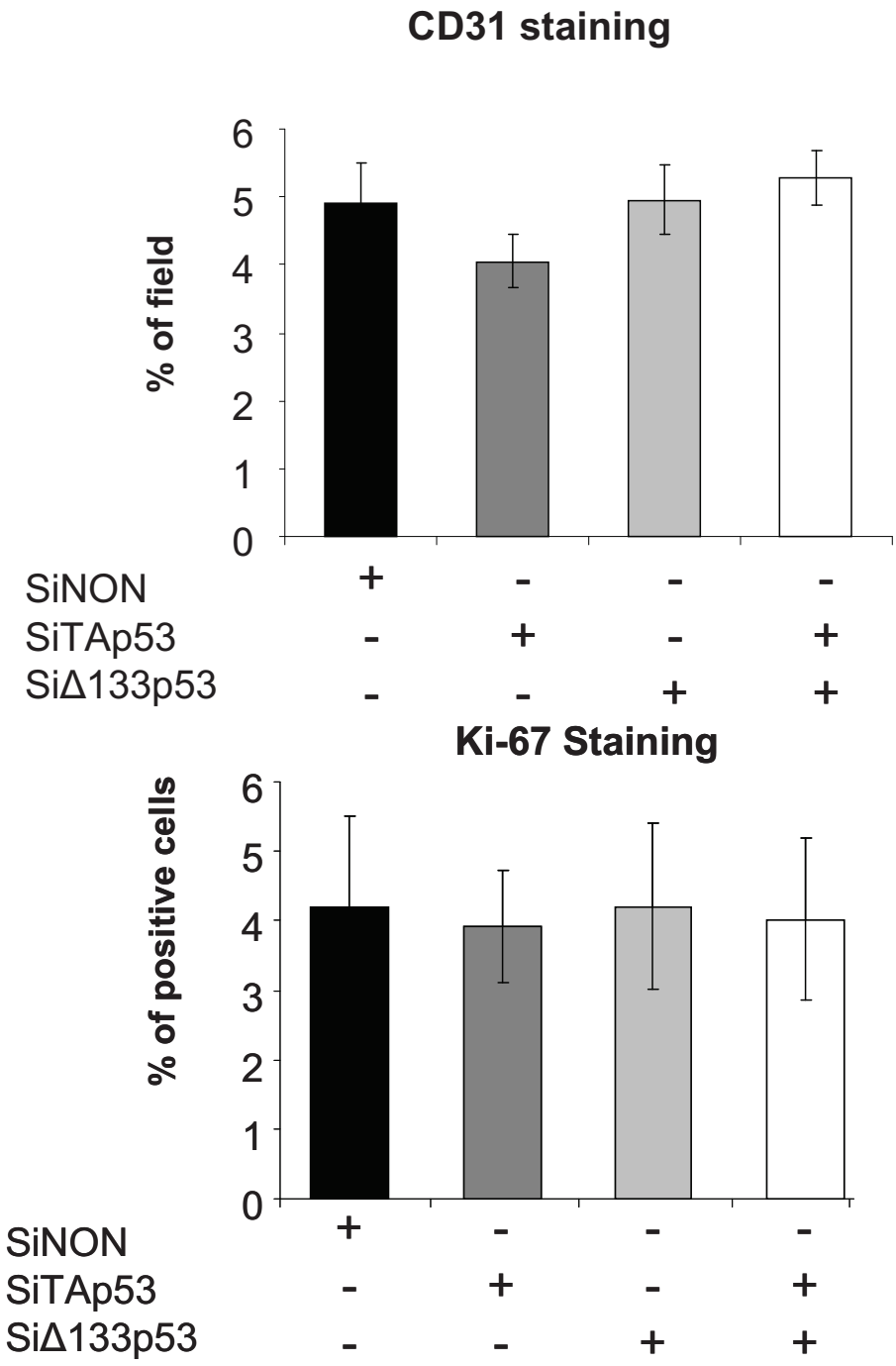


Figure 40. Quantification of immunostaining for angiogenesis index and Ki-67 for proliferation index in glioblastoma from cryosections of murine xerographs of U87 cells. For each conditions at least four samples were counted.

F.VII. Significance of the Angiogenesis Taqman Low Density Array.

The absence of involvement of FGF2 encouraged us to perform the taqman low density array – TLDA – described in the article. In this part I will discuss more in details the results obtained by the TLDA.

For this study I used the Human Taqman Angiogenesis array – provided by Applied Biosystem – which is a set of 94 genes contributing in angiogenesis or revealing a certain angiogenic phenotype, plus 2 controls. Present in this array are 28 proangiogenic and 37 antiangiogenic factors that are produced by many normal and tumour cell types. Of course this is not an exhaustive list but it contains many important precursors and factors allowing getting a relevant map of the angiogenic transcriptome. 10 markers of angiogenesis relate genes have been shown to be correlated to act upon angiogenesis and 14 targets of angiogenesis are receptors involved in angiogenic pathways. These genes are expressed in endothelial cells and thus are able to reflect the activation of these cells. There are also 5 markers of lymphangiogenesis.

When I analysed the results I was first surprised by the strong difference in the number of pro and antiangiogenic genes expressed in U87 cell line. Indeed a large majority of antiangiogenic genes was not expressed or expressed in level so low that it could not be detected by TDLA and on the contrary the majority of proangiogenic genes was expressed – Figure 27A. It is not possible to make comparison at this stage as there is only one cell line observed. However, Buckanovich *et al.* in their comparison between tumour vascular cells from ovarian cancer and vascular cells from healthy ovaries established a large profile of genes specifically expressed in tumour vascular cells (Buckanovich *et al.* 2007). In this profile 50 genes were overexpressed and 20 were newly expressed.

We decided to take into account in this assay to detect the effect of Δ133p53 on gene expression only the genes with a variation greater than 100 % – inhibition or induction – and that had a p value inferior to 0,05.

F.VII.1. Antiangiogenic factors – Fig 26B

F.VII.1.a. MMP-2

Matrix metalloproteinase 2 – MMP2 - also called gelatinaseA is the most abundant MMP and is constitutively secreted by most cells. MMP-2 degrades collagens

present in the vascular basement membrane and in the interstitium and is strongly involved in tumour angiogenesis (Fang *et al.* 2000). MMP-2 is principally an angiogenic factor through it activity of remodelling the ECM that induces cell migration and invasion, this protein is also able to cleave the plasminogen to produce angiostatin. Additionally, degradation of ECM is not necessarily correlated with angiogenesis as excessive ECM remodelling hampers and disables functions of cell motility (Jackson 2002). Particularly linked with our study MMP-2 is transcriptionnally induced by p53 (Bian and Sun 1997). As a result of Δ133p53 knockdown, p53 is induced to activate MMP-2 transcription – Figure 27 B. On the contrary we should have obtained an inhibition of transcription following p53 knockdown. Another mechanism must be involved in MMP-2 regulation when p53 is disrupted. Further investigation would be interesting to unravel this complex system.

F.VII.1.b. IL12A.

Interleukin-12α – IL-12A or p35 – is a protein that binds Interleukin-12β – IL-12B or p40 – to form the heterodimer Interleukin12 – IL-12 or p70. IL-12 has been shown to prime couples of immune cells through the production of the interferon-γ – IFN-γ – which also is responsible of the antiangiogenic activity of IL-12 (Del Vecchio *et al.* 2007). *In vivo* studies revealed that IL-12 has potent antitumour and antimetastatic activities but partly due to its antiangiogenic activity (Nastala *et al.* 1994; Tahara *et al.* 1994; Voest *et al.* 1995). IL-12 antiangiogenic activity is defined by reduction of αVβ3 integrin, VEGF-A, MMPs and augmentation of IP-10, *mig,* LMP2 and 7, between others (Del Vecchio *et al.* 2007).

In our study IL-12α seems to be tightly regulated by both p53 and Δ133p53 isoforms. Indeed in this aspect p53 knockdown induces the downregulation of IL-12α and Δ133p53 knockdown induces IL-12α's upregulation. It is quite interesting to see that Δ133p53/p53 double knockdown results in increased IL-12α mRNA levels – not significant however – that would mean that Δ133p53 effect on IL-12α expression is predominant to p53's. This result is quite in contradiction with previous work done of p53 +/+ and p53 -/- cells in mice where IL-12 levels has been correlated to p53 status although direct link has not been shown (Zheng *et al.* 2005; Tsai-Turton *et al.* 2009).

F.VII.1.c. COL4A2.

COLA42 gene is part of a big family of collagens described earlier on. Collagens 4 are the most expressed collagen type in basal membrane COL4A2 encodes Collagen alpha-2(IV) chain protein whose cleavage in C-terminus give raise to the Canstatin, an anti-angiogenic gene. Little is known however concerning its cleavage but increased collagen production is thought to enhance ECs migration and remodelling. As regards the Δ133p53 knockdown leads to COL4A2 inhibition of expression. It seems that it is depending on p53 because we cannot observe the same phenotype when p53 is disrupted. However basal levels of p53 do not enter in the regulation of COL4A2 expression but rather an induction of p53 caused for instance in our study by SiΔ133p53 treatment.

F.VII.2. Proangiogenic factors - Fig 26C

F.VII.2.a. Angiogenin

Angiogenin, a 14 kDa secreted protein bearing multiple proangiogenic facets, belongs to the ribonuclease superfamily with 33% of homology to the pancreatic ribonuclease A – for a complete review see (Gao and Xu 2008). This ribonuclease activity although weak, is essential for its proangiogenic activity. Angiogenin is also able to form a complex with ECs's α-actin involved in the conversion of plasminogen to plasmin. Plasmin in turn will be responsible for ECM and basement membrane degradation, key event for cell migration and invasion for tumour cells.

Angiogenin induces also a signal transduction pathway via its specific receptor. This 170 kDa has still not been clearly characterized but the downstream events imply ERK1/2, protein kinase B AKT and Stress Associated Protein Kinase /c-jun N-terminal kinase – SAPK/JNK. These intracellular pathways are involved in the induction of cell proliferation by Angiogenin. In fact, expression of the 170 kDa receptor is allowed only when cells are not confluent, triggering cell proliferation. On the contrary, binding to α-actin occurs when cells are close to confluence and thus is a signal for the remodelling of the basal membrane and EC migration.

Finally, Angiogenin is also capable to induce rRNA transcription via its translocation to nucleus by an ANG-binding element.

The strong inhibition of Angiogenin in our study by SiΔ133p53 treatment in the U87 cell line is independent of p53. This factor is then a good candidate for the explanation of the blockade of tumour growth. Additionally, the separate pathways involved in cell migration and cell proliferation can suggest us that this factor has an important role in our system – we found an effect on migration but not on proliferation of ECs. These observations strongly put Angiogenin as a determinant factor involved in Δ133p53 disruption.

F.VII.2.b. Midkine

Midkine – MDK or Neurite Growth-promoting Factor 2 – is a basic heparin-binding growth factor belonging to a family of three members, Pleiotrophin, retinoic acid heparin-binding protein and Midkine. Midkine has various actions in anti-apoptosis, mitogenesis, transforming, tissue repair, inflammation, angiogenesis and fibrinolysis, and is expressed during embryogenesis but extremely repressed in adults. It is then overexpressed in tumours and is deeply involved as a marker of carcinomas (Dai 2009).

In our study Midkine is downregulated by SiΔ133p53, dependently of p53.

F.VII.2.c. Hepatocyte growth factor

Scatter factor/hepatocyte growth factor and its receptor tyrosine kinase c-met are involved in processes but particularly in tumour growth and angiogenesis (Abounader and Laterra 2005). Likewise other master regulators of angiogenesis such as FGF-2 and VEGF, HGF displays various means of actions but giving a complete set of them upon angiogenesis is not the subject at this point of investigation. As its strong involvement in angiogenesis, HGF regulation could appear of importance. However the mild induction observed in must be taken cautiously, although significant.

F.VII.2.d. VEGF-C

As described previously, VEGF-C is mainly involved in lymphangiogenesis although it controls also formation of the vasculature. In our context VEGF-C mRNA expression is responsive to p53 knockdown. These interesting results suggest that VEGF-C and p53 have an unexpected link, indicating a novel argument for p53 to control tumour related lymphangiogenesis, tumour dissemination and metastasis.

F.VII.2.e. Pleiotrophin

Pleiotrophin – PTN – is the second member of a family of structurally related heparin-binding growth factors. Pleitrophin is a cytokine in which binding to heparin is a critical event permitting the binding to its receptor and its inhibition, the receptor protein tyrosine phosphatase – RPTPβ/ζ. This receptor dephosphorylates β-catenin, β-adducin, Fyn, GIT1-Cat and P190RhoGAP, ALK kinase and other receptor tyrosine kinase (Perez-Pinera *et al.* 2007). Pleiotrophin is a important activator of the angiogenic switch by initiating endothelial cell proliferation, migration, tube formation and regulates the levels of FGF-2 and VEGF-A.

The fact that p53 is involved directly an indirectly in the regulation of Pleiotrophin and Midkine, two homologs of the same family allow to hypothesize that there is a conserved element in their promoter targeted by p53 and Δ133p53.

F.VII.3. The angiogenic marker ANGPTL4 – Fig 26D

Angiopoietin-like 4 protein is part of the subfamily of angiopoeitin-like proteins which contributes in many functions like glucose and lipid metabolism, immune response and atherosclerosis. Angiopoietin-like protein 4 is induced under ischemic and hypoxic conditions suggesting a role in angiogenesis. It has also implications in cell migration, invasion and angiogenesis, tumour dissemination and metastasis (Stapleton *et al.* 2010). However the exact role of this protein in angiogenesis is still controversial as several studies contradict each other. (Le Jan *et al.* 2003; Cazes *et al.* 2006; Yang *et al.* 2008; Tian *et al.* 2009; Ma *et al.* 2010).

ANGPTL4 is deeply involved in angiogenesis and regulation of its expression means a modification of angiogenic potency. The contexts in which ANGPTL4 clearly enhance or inhibit angiogenesis is still too elusive to conclude.

In our context ANGPTL4 is strongly inhibited in response to SiΔ133p53 and so would indicate that constantly with our results, ANGPTL4 exerts a proangiogenic activity. This modulation seems also to be dependent of p53 as the double siRNA show a milder inhibition of this factor. In conclusion this factor, although not clearly characterized, is of strong importance in our study.

CHAPTER 2:

Regulation of FGF-2 by p53

A. p53 and translation

A.I. Mechanisms of translation initiation.

Translation is a master regulatory mechanism of protein synthesis involving the steps of initiation, elongation and termination. However it is generally thought that translation initiation constitutes the main step of translation regulation. In the following paragraphs I will only focus on this process describing two main translation initiation mechanisms that are the Cap dependent translation initiation mechanism – 5'end scanning translation initiation – and the Internal Ribosome Entry Site mechanism – IRES translation initiation.

The machinery involved in all types of translation initiation mechanisms is based on a pool of constitutive factors that participate in a two steps program: the attachment of the 43S pre-initiation complex and the encountering of the start codon which signals the formation of the 80S ribosome and start of translation. I will describe it briefly – compared to its enormous complexity and the amount of data available – considering Cap-dependent translation as the template mechanism and then comparing it to IRES-dependent translation - for more information see the review (Jackson *et al.* 2010).

A.I.1. Cap dependent translation initiation.

Most eukaryotic mRNAs undergo this mechanism to deliver protein s to the cell. As a dynamic and continuous mechanism the assembling of 43S pre-initiation complexes involves either synthesis of ribosomosal proteins and the 18S ribosomal RNA, forming the 40S subunit, eukaryotic initiation factors – eIFs – and complementary factors, or the recycling of 80S post-termination ribosomal complexes dissociated into subunits. The 43S pre-initiation complex more specifically comprises, besides the 40S subunit, a set of eIFs engaged in the presentation of the Met-tRNA initiating anti-codon – eIF2-GFP-Met-tRNA ternary complex or eIF2TC plus co-activating factors such as eIF3, eIF5 and eIF1 involved in ribosome scanning as well.

The 43S pre-initiation complex is next recruited by the eIF4F cap-binding complex composed of the eIF4E which binds to the m7GpppG 5′ terminal 'cap' structure of mRNA, the eIF4A mRNA helicase and eIF4G which enhances helicase activity. Additionally, two other mRNA helicase catalysers – eIF4B and H – can help the eIF4A protein and the Polyadenylation Binding Protein – PTB – joins the 3' mRNA

end to the eIF4F complex, constituting a loop-like structure. After binding of the 43S complex to eIF4F to form the 48S complex, mRNA is unwound, which undoes secondary structures, and scanned from the 5' end in direction to the 3' end until it founds a start codon, in the same time eIF4E sticks to the cap structure of the mRNA and unbinds the eIF4F complex. The more secondary structures the 5'end have, the more ATP is needed for their unwinding.

There are canonical and non canonical initiation codons. The canonical codon is AUG and with best extend GCC(A/G)CC**AUG**G – the Kozak sequence, and non canonical codons are CUG or UUG in eukaryotes. When the 48S subunit meets the initiation codon, it stops and triggers a mechanism that recruits the 60S subunit. In this role eIF1A plays an important role by maintaining recognition specificity. The open scanning-competent conformation of the 40S complex switches to a closed and locked codon-anticodon base pairing conformation through tightening its interaction with eIF1A and displacing eIF1 from the P-site – the peptidyl tRNA binding site on the ribosome. This move discards the repression of eIF1 upon eIF5 which is an eIF2-specific GTPase-activating protein – GAP – leading to the dissociation of eIF-2-GDP from tRNA and partial release from the 40S subunit, GTP hydrolysis and Pi release. Following that the 60S subunit of the ribosome – composed of a 5S RNA, a 28S RNA , a 5.8S subunit and ribosomal proteins – plus eIF5B-GTP binds to the 40S complex and replace the others eIFs – eIF1, eIF1A, eIF3 and eIF2-GDP. Translation can proceed (Jackson *et al.* 2010).

A.I.2. IRES dependent translation initiation.

IRESs, first discovered in uncapped viral mRNAs, are long highly structures composed of secondary and tertiary structures in the 5'end mRNA. Intensive research has yield to consequent lists of IRESs present in viral as well as cellular mRNAs that can be found for instance at www.rangueil.inserm.fr/**iresdatabase/** or http://iresite.org/ (Bonnal *et al.* 2003; Mokrejs *et al.* 2010). Despite the consequent number of discovered cellular IRESs, their uniqueness of structure renders difficult the setting up of common features, common co-factors and solid consensus mechanism of translation initiation (Baird *et al.* 2006). For this reason, IRESs are mostly described by the conditions that induce their activity or on the contrary phenotypes resulting from their activation. IRES are present in proto-oncogenes, growth factors, receptors, and transcription factors, have

strong tissue specificity and are involved in many conditions such as apoptosis, cell cycle, development, hypoxia, ischemia, heat shock, myogenesis, ER stress and more.

The identification of IRESs is strongly challenged by stringent and complete set of control experiments to avoid the possibility of cryptic promoters, aberrant splicings or even sequences able to induce mRNA cleavage. In fact, couples of the discovered IRESs have been re-questioned, lightening up the difficulty to prove the functionallity of such structures (Hellen and Sarnow 2001; Kozak 2005; Baranick *et al.* 2008).

IRES-dependent initiation of translation is still not a clearly definied mechanism due to the specificity of each IRES element. The 40S ribosomal subunit recognizes a specific feature in the 5'UTR of the mRNA and is directly recruited or recruited by IRES Trans-Acting Factors – ITAFs – such as ribosomal proteins, eIFs, miRNAs or other proteins (Fitzgerald and Semler 2009). In this process the eIF4F complex is not implied and most of the time no scanning mechanism neither occurs, impeding Kozak's hypothesis of general mechanism of translation initiation. Numerous co-regulators of IRES activity mentioned above as ITAFs – PTB, unr, hnRNP C1/C2, hnRNP A1, hnRNP K, La antigen, DAP5, p53nrb etc… – are thought to give some more information on the IRES initiation of translation. Strikingly ITAFs can shuttle between nucleus and cytoplasm like hnRNP A1, which is involved in human rhinovirus type 2 RNA – HRV-2, human apoptotic peptidase activating factor 1 mRNA – apaf-1 – and FGF-2 mRNA. Additionally, some hnRNPs like hnRNP A1 have been found in transcriptional complexes, associated to promoter (Paramasivam *et al.* 2009). These kind of evidences led us to hypothesize cross talks between transcription and translation mechanisms. As regards, we found a coupling mechanism of FGF-1A promoter/mRNA expression during muscle differentiation through the presence of a *cis*-acting sequence in the promoter A allowing specifically the induction of the FGF-1A IRES (Conte *et al.* 2009).

IRES dependent translation is generally activated when the cell undergoes some stress conditions – listed above – in which a least in a part of them, cap-dependent translation in down regulated. IRESs allow the cell to overcome general translation downmodulation and to produce smart, precise and rapid blow of specific factors that will help the cell to respond to a stringent context. There are several mechanisms of cap dependent translation inhibition either induced by viruses or through regulation of eIFs availability and activity. For instance, enteroviruses and rhinoviruses are single strand RNA viruses and possess IRES elements to drive their translation. Not only their RNA

is directly translated after viral infection, but they also encode proteases that cleave eIF4G and PABP proteins, inhibiting cap-dependent translation. Levels of general cap-dependent translation are also governed by a co-factor of eIF4E protein, eIF4E-binding protein – eIF4E-BP1. Under certain stresses or EncephaloMyoCarditis virus – EMCV – infection, this protein is underphosphorylated and able to fix eIF4E and thus inhibits eIF4F mediated translation initiation. Additionally, under endoplasmic reticulum – ER – stress, a double-stranded RNA-activated protein kinase-like ER kinase – PERK – is shown to be activated in response to ER stress and to phosphorylate eIF2α-subunit – one of the three subunit of the eIF2 protein, thereby inhibiting cap-dependent translation initiation (Harding *et al.* 1999).

A.II. p53 regulates translation

As mentioned in the introduction, p53 mainly acts through the regulation of transcription activity, but also through direct interaction with other proteins. In this part I will focus on its barely known ability of binding and regulating mRNA translation.

The mammalian target of rapamycin – mTOR – is critically involved in regulation of translation and many other processes like autophagy, cell proliferation and cell survival. To emphasize the importance of mTOR, it is to note that proteins that inhibit mTOR are often considered as tumour suppressors. Fen *et al.* have shown that p53 activation induces mTOR inhibition and consequently the dephosphorylation of eIF4E-BP1 and p70S6 kinase (Feng *et al.* 2005). Generally, mTOR activation induces p70S6 kinase activity that in turn will phosphorylate the S6 ribosomal protein, a co-activator of protein synthesis. The first team who discovered the regulation of these proteins by p53 was of Clemens et al, whose work will be discussed in more in details below (Horton *et al.* 2002). These findings show that p53 is able to inhibit both cap-dependent translation initiation and IRES-dependent translation initiation.

Translation inhibition by p53 is not restricted to the messenger class but also concerns ribosomal and transfer RNAs. Indeed, it regulates the activity of RNA polymerases I and III, potentially affecting the synthesis of components of the translational apparatus (Chesnokov *et al.* 1996; Cairns and White 1998; Budde and Grummt 1999). Furthermore, p53 can associate with ribosomes through covalent binding to the 5.8 rRNA (Fontoura *et al.* 1997).

The group of Clemens *et al.* has worked for several years on the interaction of p53 and eIF4E-BP1 (Horton *et al.* 2002; Constantinou *et al.* 2003; Clemens 2004;

Tilleray *et al.* 2006; Constantinou and Clemens 2007; Constantinou *et al.* 2008). They have shown that independently to the apoptotic pathway, thermosensitive p53 – p53ts – induces translation inhibition through dephosphorylation of eIF4E-BP1 and p70S6, and cleavage of eIF4G1 form and eIF4B, a phenomenon that induces cell death if p53ts overexpression lasts more than 16h. These authors have also shown this phenomenon in an inducible wildtype p53 context correlating with dephosphorylation and accumulation of eIF4E-BP1. Lately they have found that p53 induces irreversibly the truncation of eIF4E-BP1 which becomes more stable, binds eIF4E and target it to the proteasome.
p53 is able to specifically target and modulate the translation of several mRNAs at their 5'UTR such as its own mRNA, FGF-2 mRNA and cyclin dependent kinase 4 – cdk4 (Ewen *et al.* 1995; Mosner *et al.* 1995; Galy *et al.* 2001; Galy *et al.* 2001). Particularly, our group has demonstrated that p53 is able to modify the structure of the 5'UTR of the FGF-2, leading to an inhibition of IRES activity. With FGF-2 5'UTR leader-CAT chimeric constructs, we have shown that this regulation is specific to the wild type p53 but not DBD mutants. Interestingly a p53 mutant the for transactivating domain is still able to repress FGF-2-CAT expression, suggesting that transcriptionnally disabled p53 can act upon FGF-2 translation. In this respect p53 – or its isoforms – can be considered as authentic ITAFs. Consistent with these results, Oberosler *et al.* demonstrated that in *in vitro* experiments p53 binds RNA as well as DNA and has strong RNA-RNA annealing activity suggesting another argument in favour of p53 activity over translation (Oberosler *et al.* 1993; Nedbal *et al.* 1997).

All these findings allowed us to better understand the mechanisms controlled by p53. In fact the double layer of transcription-translation regulation corresponds to a smart answer of the cell to stress conditions thanks to p53. After a short and mild stress, the overall machinery of translation is halted diminishing the energy necessary for protein production and p53 induces a cell cycle arrest response. When on the contrary stress conditions last longer, p53 engage apoptosis pathways and irreversible translation arrest.

Overexpression of p53 has couples of effects upon protein expression: it first inhibit translation through eIF4E-BP1, but it also induce the transcription of several target genes and thus their protein expression. Additionally, p53 properties towards mRNA show that it is able to inhibit specific IRES-dependent translation. In the respect to these evidences, we hypothesized that p53 could act on a more significant pool of mRNAs and modify their translation as well as their transcription.

B. Regulation of FGF2 expression by p53

B.I. Translational control of p53

In this study I used U2OS cells, a wildtype p53 osteosarcoma cell line. The advantage of this strain is, apart from its potential of growth and the easiness of cell culture, its easiness to be stably transfected by plasmids.

To study the rates of cap-and IRES-dependent translation a specific tool has been developed in the laboratory: the "Lucky Luke" bicistronic vector. In our particular situation, this vector possesses from 5' to 3' a constitutive and strong promoter, the Renilla Luciferase Open Reading Frame – ORF, the sequence of IRESs or hairpin sequence – inhibiting the ribosome to restart translation at second cistron, the Firefly Luciferase ORF and the β-globin 3'untranslated region containing the polyadenylation site. The following figure 41 presents a schematic view of the bicistronic vector and the different plasmids used and constructed.

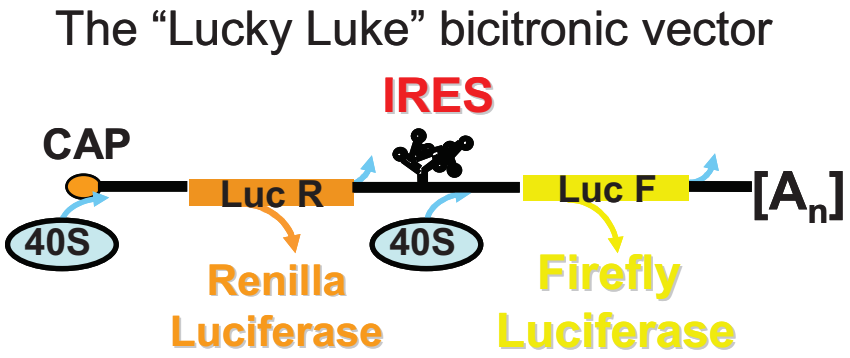


Figure 41. Schematic representation of the "Lucky Luke" bicistronic plasmids. This vector allows the study of cap-dependent and IRES-dependent translation: The first Renilla Luciferase – LucR – is under cap-dependent translation whereas the Firefly Luciferase – LucF – is under IRES-dependent translation.
Plasmids used for the experiments – Constructed by L. Créancier (Creancier *et al.* 2000; Creancier *et al.* 2001).
pCRHL: CMV promoter, LucR ORF, Hairpin sequence, LucF ORF
pCRFL: CMV promoter, LucR ORF, Human IRES FGF-2 sequence, LucF ORF
pCRMP2L: CMV promoter, LucR ORF, Human IRES C-myc sequence, LucF ORF

Because transient transfection in cells occurs only in a part of them, and yields non physiological levels of transgene expression, we decided to select pools of stable cell lines expressing each construction mentioned above. This provides the advantage of a homogenous population expressing at least one copy of transgene per cell that equally map the whole genome of the strand. However amplification results in selection of the

most potently dividing cells – that for instance have integration in a gene that repress cell growth – to the expense of the other cells. Consequently I constructed three stable cell lines expressing the bicistronic vector mentioned above.

Finally, in these selected cell lines, I did some siRNA transfections. Results are presented below – Figure 43 and 42.

In the first experiments I used bicistronic vectors with CMV promoter and either FGF2 IRES or c-myc IRES mentioned above – Figure 41. Indeed, c-myc functioning as an oncogene and consequently in opposition to p53, the c-myc IRES, as well as the FGF2 IRES, seemed us a good candidate for translational regulation by p53.

Here we show that knockdown of p53 induces an augmentation of FGF-2 and c-myc IRES activities. Scrumble siRNA or various doses of Sip53 – targeting the exon 7 – presented different firefly/renilla luciferases ratios. At low doses knockdown of p53 – 15 and 30 nM – induce an increase in FGF-2 and C-myc IRES mediated translation but not in the Hairpin control. At high doses the same effect persists for the FGF-2 IRES but is inversed for c-myc IRES. Consequently the levels of p53 are important and can totally induce opposite responses.

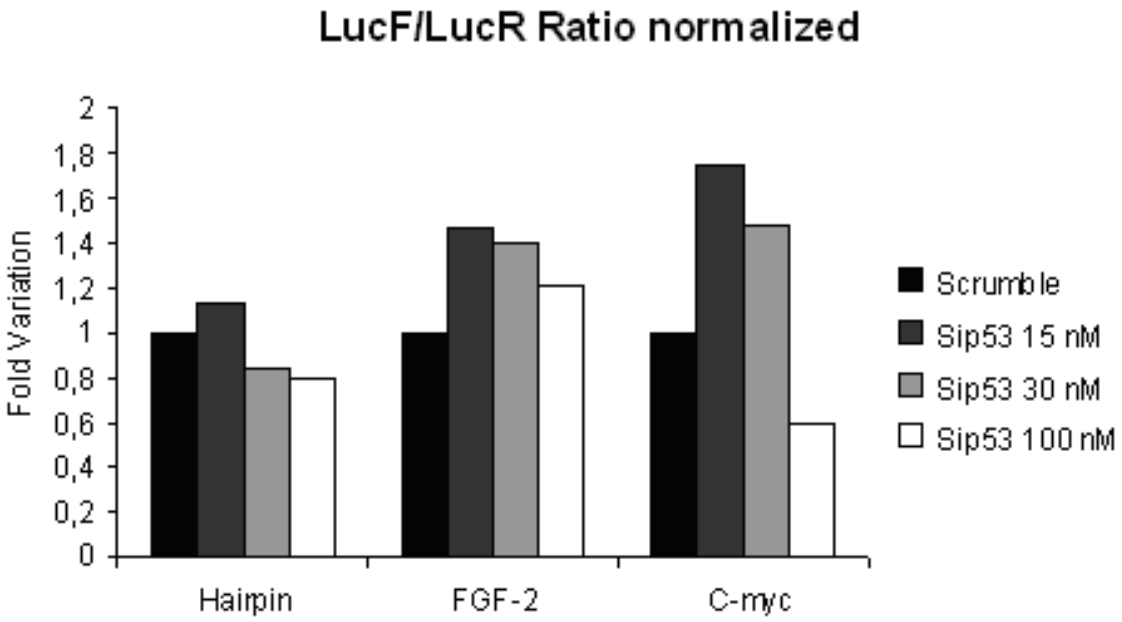


Figure 42. IRES activity normalized in U2OS stable cell lines treated by different concentrations of Sip53 or Control SiRNA. Absolute values of IRES activities show that Hairpin activity in more than 10 times lower to other IRESs.
Samples are lysed in Passive Lysis Buffer 1x and Luciferases luminescences are measured with a LB 960 Centro microplate luminometer –Berthold France SAS, Thoiry, France - using the Dual-Luciferase Reporter Assay System – Promega, Charbonnières-les-Bains, France. Experimental procedure is following the manufacturer's indications. Experiment repeated twice in duplicate and Mean is represented

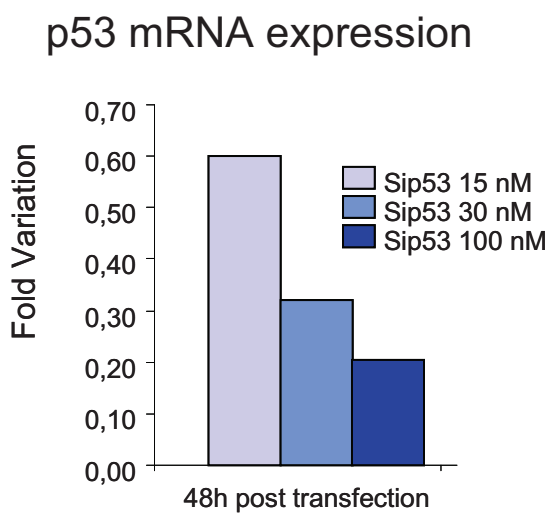


Figure 43. p53 mRNA assessment U2OS cells following SiRNA treatment using QRT-PCR. Sample quantity is normalized with 18S rRNA using the $2^{-\Delta\Delta Ct}$ method and set to percentage of control. Data presented are fold variation to SiScrumble Control SiRNA. Sip53 SiRNA sequence: 5'GACUCCAGUGGUAAUCUACd[T]d[T] (Brummelkamp *et al.* 2002)

B.II. FGF-2 endogenous regulation by p53

In 1994, Ueba *et al.* demonstrated that overexpression of wildtype p53 induced repression of FGF-2 transcription through the binding to its promoter between -20/+50 in U87MG and SKHepG2 cell lines. In the mean time they showed that the mutant p53 induced FGF-2 transcription (Ueba *et al.* 1994). The protein encoded by this p53 mutant – 143Val-Ala – can still bind to the p53 DNA consensus element and can retain some of the transactivation properties of wild-type p53 (Funk *et al.* 1992; Zhang *et al.* 1993; Friedlander *et al.* 1996). In some particular sets it is even able to inhibit tumour growth (Simpson *et al.* 2005). However observations linking p53 transcriptional regulation and FGF-2 haven't been confirmed elsewhere.

On another hand, our laboratory has proved that p53 is also able to regulate the translation of FGF-2 using FGF-2-CAT chimeric constructs (Galy *et al.* 2001; Galy *et al.* 2001). However the endogenous levels of FGF-2 were not investigated. To answer this question we decided to analyse this aspect.

B.II.1. p53 knockdown

Surprisingly Sip53 treatment induced in U2OS cells a decrease of FGF-2 protein levels. This is shown in U2OS stably transfected cells or in native U2OS. This decrease was not correlated with a significant decrease in FGF-2 mRNA levels – Figures 44, 45 and 46. In a previous work we ruled out the possibility of a decreased stability of FGF-2-CAT constructs when cells were overexpressing p53 (Galy *et al.* 2001). Δ133p53 does not seem to participate in this regulation as in none of the cell lines it changed protein or mRNA levels. As we can see in U2OS cell line SiΔ133p53 treatment induced an overexpression of p53. At this point of the investigations we did not go further and did not analyse target genes of p53.

These results are in contradiction to our previous results and to the work of Ueba *et al.* It can be argued first that in both studies mentioned above, none of them worked with the endogenous proteins but with plasmid constructions overexpressing p53 or chimeric constructions. Secondly and more importantly, in their cases they mimicked a stress resulting in p53 overexpression contrarily to my experiments. This may unravel specific action of p53 as its levels are thought to oscillate during cell cycle (Tyson 2006). To answer this question we then decided to induce p53 expression.

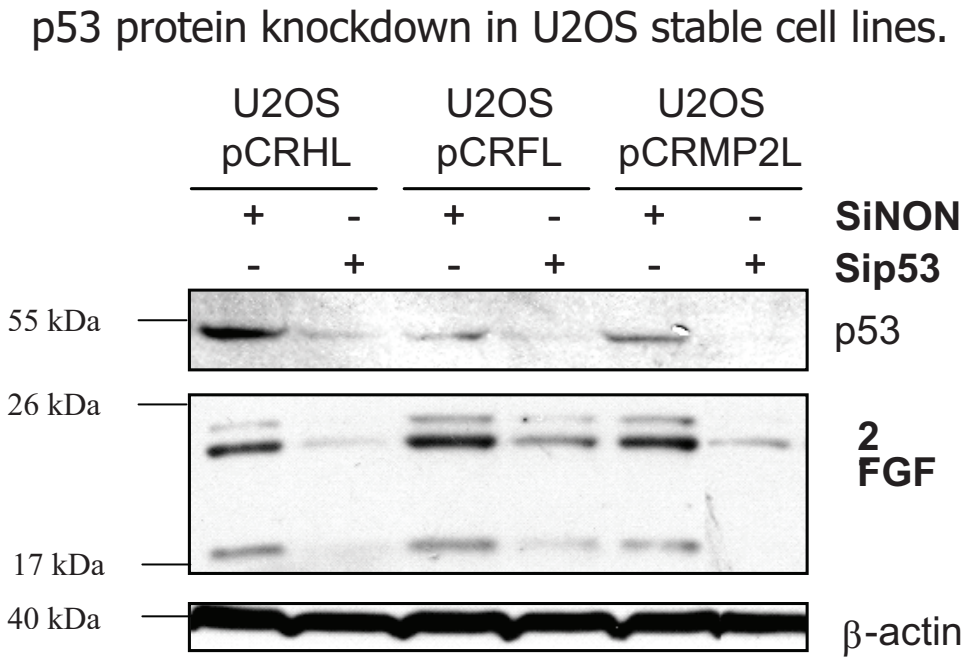


Figure 44. Western blot showing p53 and FGF-2 protein expression following sip53 transfection in U2OS stable cell lines.. p53 immunostaining with Sapu antibody and β-Actin with β-Actin antibody and FGF-2 with sc-79 antibody – Santacruz Biotechnologies, Heidelberg, Germany.

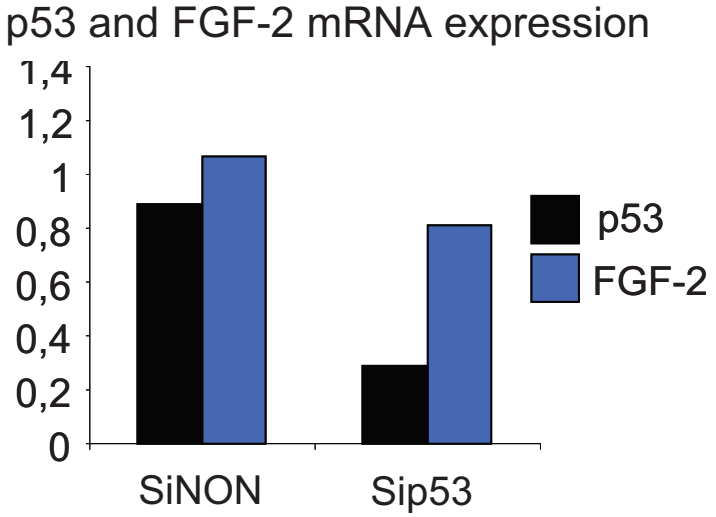


Figure 45. FGF-2 and p53 mRNA quantification in U2OS cells following SiRNA treatment using QRT-PCR. Experiments were repeated twice in duplicate and mean is represented. Sample quantity is normalized with 18S rRNA using the $2^{-\Delta\Delta Ct}$ method and set to percentage of control. Sequence of SiRNAs are in Suppl. Table II. FGF-2 sense primer: CATCAAGCTACAACTTCAAGCAGA ; Anti-sense primer : CCAGTAATCTTCCATCTTCCT, obtained with Primer Express3.0

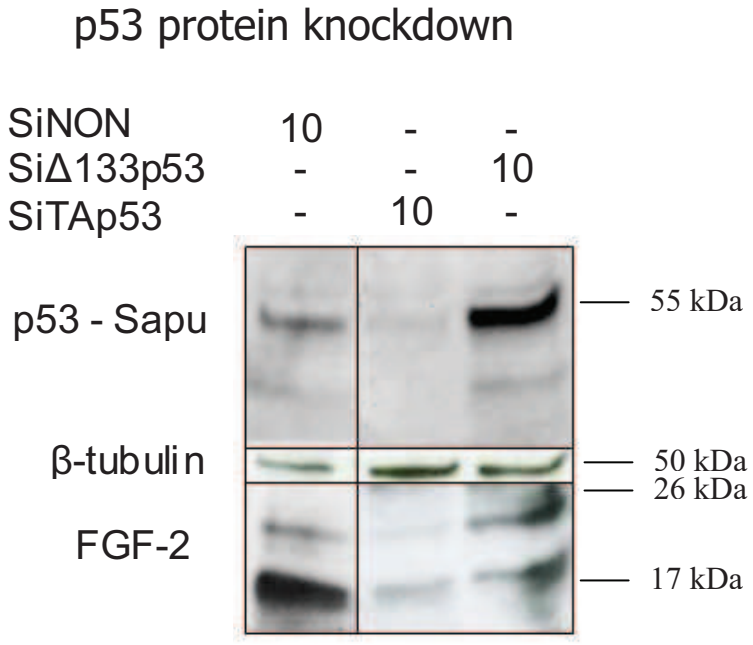


Figure 46. Western blot showing p53 and FGF-2 protein expression following sip53 transfection in U2OS cells. .. p53 immunostaining with Sapu antibody and β-Actin with β-Actin antibody and FGF-2 with sc-79 antibody

B.II.2.Response to p53 induction

B.II.2.a. Actinomycin D

We first use Actinomycin D – ActD – to induce p53 at 47 nM. ActD is used both for basic research and clinical research as a transcription inhibitor at high doses and also as a chemotherapeutic agent and formerly an antibiotic. Its toxicity is due its ability to bind DNA at sites of transcription initiation, rich in sequences GC but also RNA polymerase I and II (Sobell 1985). Through binding of RNA pol II ActD induce the activation of p53 and at some doses of ActD transcription inhibition can be avoided, leading to only p53 activation and cell cycle arrest (Choong *et al.* 2009). To note in our experiment the dose used for treatment is a high dose that can induce transcription inhibition.

Following ActD treatment we can observe a double effect, one dependent to p53 and the other that might result from RNA synthesis inhibition – Figures 47 and 48. As we previously observed, knockdown of p53 induce FGF-2 protein decrease, but when ActD is added and consistently with time it inhibits FGF-2 mRNA and protein presence – here is shown mRNA levels only after six hours of treatment.

However it is quite difficult to interpret with ActD treatment as p53 activation is supposed from my previous experiments to induce FGF-2 protein levels and ActD inhibits FGF-2 transcription expression. To get rid of the transcription parameter, we decided to use doxorubicin

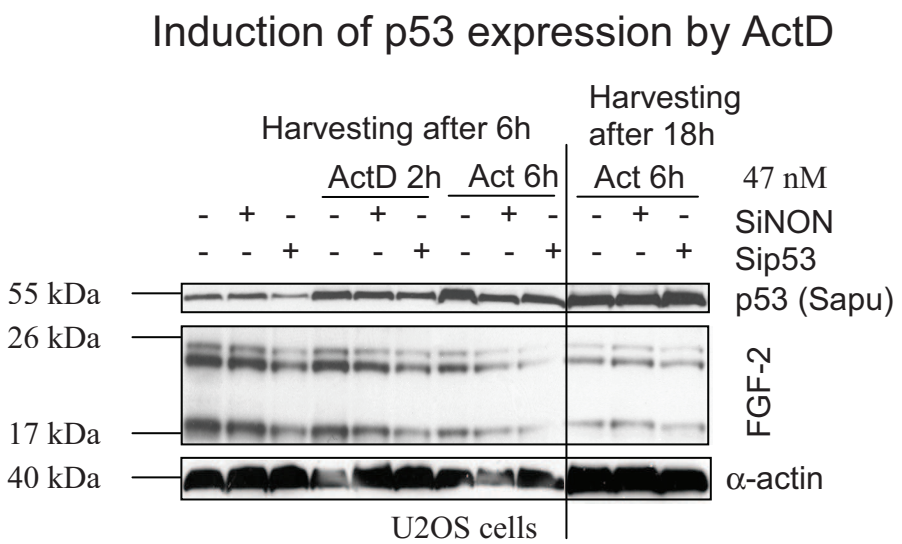


Figure 47. Western blot showing p53 and FGF-2 protein expression following ActD treatment in U2OS cells. .. p53 immunostaining with Sapu antibody and β-Actin with β-Actin antibody and FGF-2 with sc-79 antibody

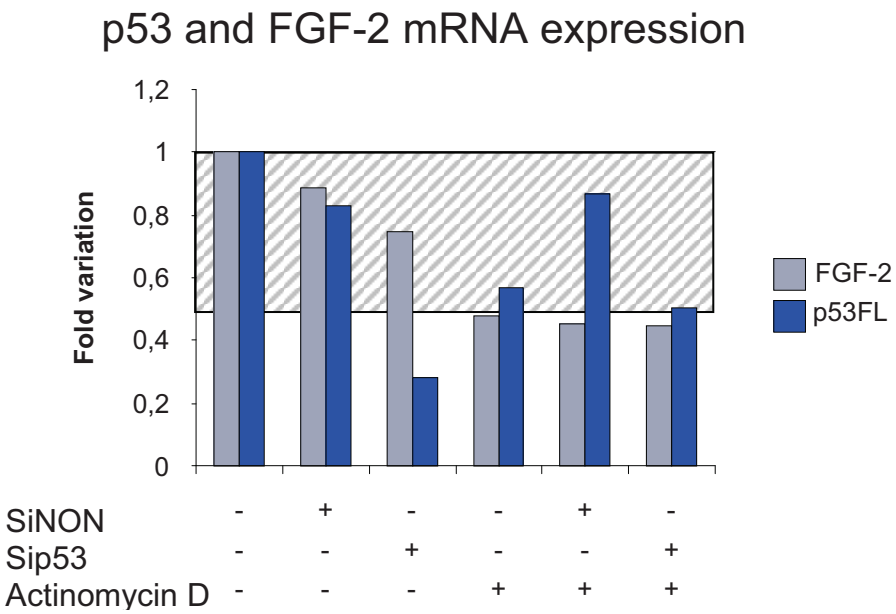


Figure 48. FGF-2 and p53 mRNA quantification in U2OS cells following ActD treatment using QRT-PCR.

B.II.2.b. Doxorubicin

Doxorubicin – Dox– is of great use in chemotherapy although it has secondary effects such as heart injury. To overcome this issue liposomal derivatives have been engineered. As ActinomycinD its use must be tightly titrated to have best effects. Dox mode of action is intercalating DNA resulting in its impairment and its damage. It also inhibits the topoisomerase II and produce reactive oxygen species. For all these reasons Dox potently induces apoptosis. To note Dox does not induce transcription inhibition on the contrary of ActD.

In this respect we induced two types of stresses: one short stress followed by a time of recovery or continuous stress until harvest. We have previously shown that short Dox stress does not induce apoptosis but rather cell cycle arrest so we wanted to see if the regulation by p53 was a survival response or an apoptotic one. We used Dox at the common dose of 1 µM.

After dox treatment, FGF-2 rapidly decreases but then is expressed again to make up the levels of expression of the control – Figure 49 and 50. This shows a double mechanism is which FGF-2 synthesis is first blocked and then after longer treatment is actively produced. As FGF-2 half life in solution is quite short – few hours, accumulation of FGF-2 can not be considered in this experiment (Nur *et al.* 2008) . mRNA levels assessment shows us that no significant variation of FGF-2 or p53 mRNA expression is observed. Regarding the treatment in its self, we can see there is no difference between a short stress followed by recovery – inducing cell cycle arrest – or continuous Dox treatment – inducing apoptosis. This means that the variations of FGF-2 protein levels can not be associated with an apoptotic stress but is more generally triggered by doxorubicin stress.

However doxorubicin mean of action is not mediated by p53: for instance, Sphingosine Kinase 2 and the transcription co-factor FHL2 are involved in dox induction of p21 and cell death, independently to p53 (Martin *et al.* 2007; Sankala *et al.* 2007). Dox also induces ROS independently to p53 and induce apoptosis through Bax and Bcl-A modulation in p53 null cells SaoS-2 (Tsang *et al.* 2003). It is thus important to know if the downmodulation of FGF-3 we have observed is not due to a p53-independent response.

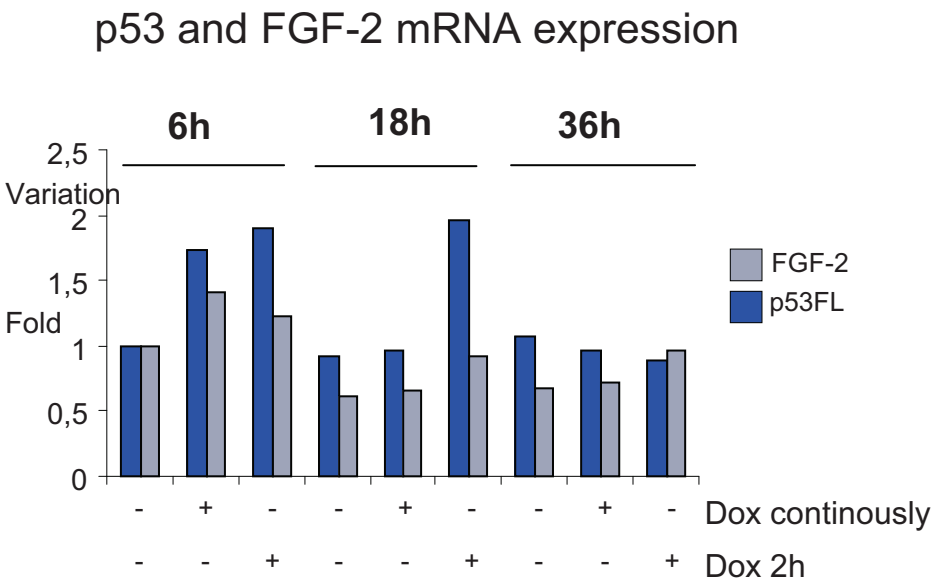


Figure 49. FGF-2 and p53 mRNA quantification in U2OS cells following Dox treatment using QRT-PCR.

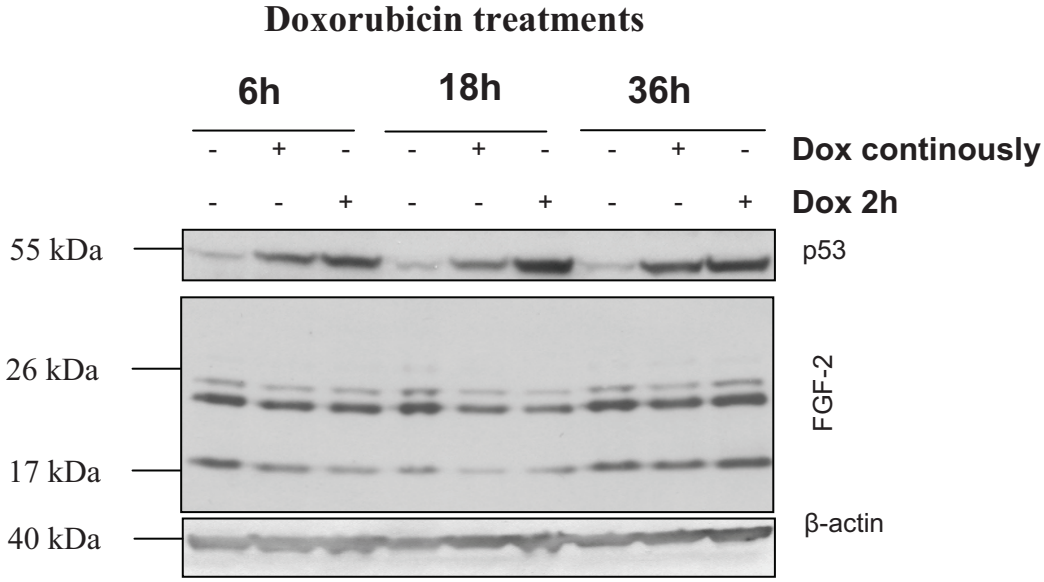


Figure 50. Western blot showing p53 and FGF-2 protein expression following Doxorubicin treatment in U2OS cells. .. p53 immunostaining with Sapu antibody and β-Actin with β-Actin antibody and FGF-2 with sc-79 antibody

BII.2.c. Overexpression of p53

To overexpress p53 in U2OS cells I used a Replication-defective adenovirus serotype 5 expressing p53 – Gift from P. Roberston – SuMO, Dundee. These viruses have the advantage to be strongly expressed in all types of cells.

In order to better understand the dynamics of FGF-2 regulation by p53, I harvested my cultures at different times after infection. The results are presented in the Figure 51 and show the protein expression of p53 and FGF-2 in U2OS cells.

As we can see, overexpression of p53 in U2OS did not have strong effect on FGF-2 protein expression – Figure 51. Even with strong expression of p53 aftr 36h of treatment, which should kills most cells, and apparently induces the proteolysis of p53 into truncated forms of p53, no consistant variation of FGF-2 can be observed. For this reason, we concluded that if p53 is involved in the stress mediated inhibition of FGF-2 protein expression, it is through modifications of p53 that enables it to bind FGF-2 promoter.

Therefore these results show us a complex mechanism in which induction of a p53 dependent stress hampers transiently induces FGF-2 expression by a mechanism still not clearly defined. As showed previously by our work and in the litterature, p53 act upon transcription, translation but also upon FGF-2 bioavaibility. For this reason we decided to analyse the secretion of FGF-2.

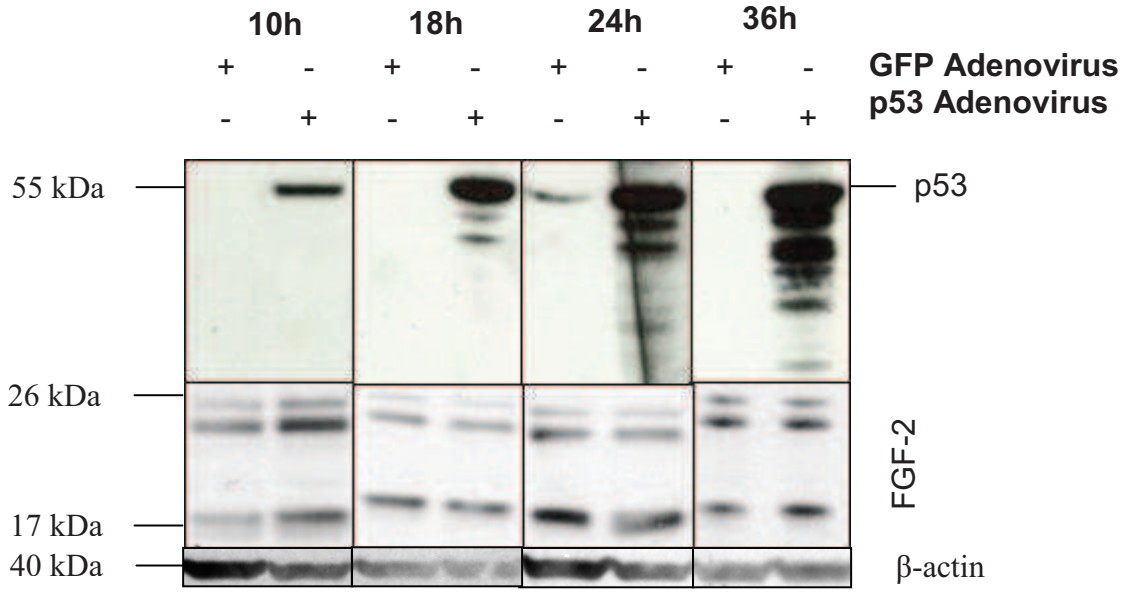


Figure 51. Western blot showing p53 and FGF-2 protein expression following adenovirus p53 infections in U2OS cells. The equivalent of a MOI of 1 was injected in U2OS media. p53 immunostaining with Sapu antibody and β-Actin with β-Actin antibody and FGF-2 with sc-79 antibody

B.II.3. FGF-2 secretion

FGF-2 secretion is not a common mechanism as FGF-2 does not have a signal peptide, and does not take any conventional route for secretion. On the contrary it involves exocytotic vesicles, the α-subunit of the Na^+/K^+-ATPase and the interaction with HSPGs (Florkiewicz *et al.* 1998; Taverna *et al.* 2003; Zehe *et al.* 2006). As p53 is involved in the bioavaibility of unbound FGF-2 through regulation of FGF-BP and heparanase expression, we wandered if modulation of p53 would modify the activity of these proteins and thus catalyse FGF-2 secretion.

As we can see knockdown of full length p53 induce less FGF-2 secreted. This is due to the decrease of FGF-2 protein levels – Figure 52. Through this ELISA in U2OS cell line, it appears that FGF-2 is not regulated by p53 through its secretion. Other techniques should be undertaken to better understand FGF-2 expression secretion and bioavaibility in the ECM such as pulse chase techniques for instance.

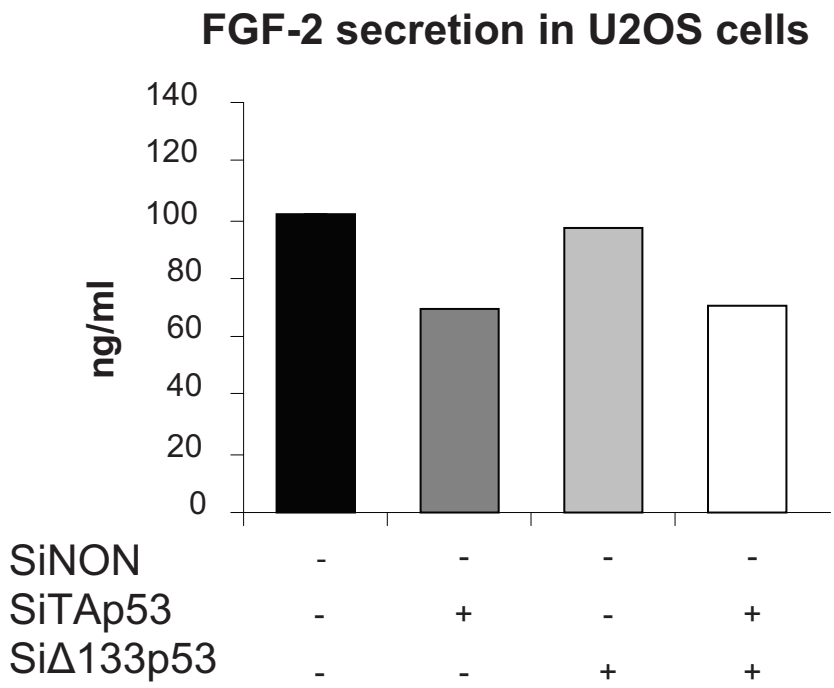


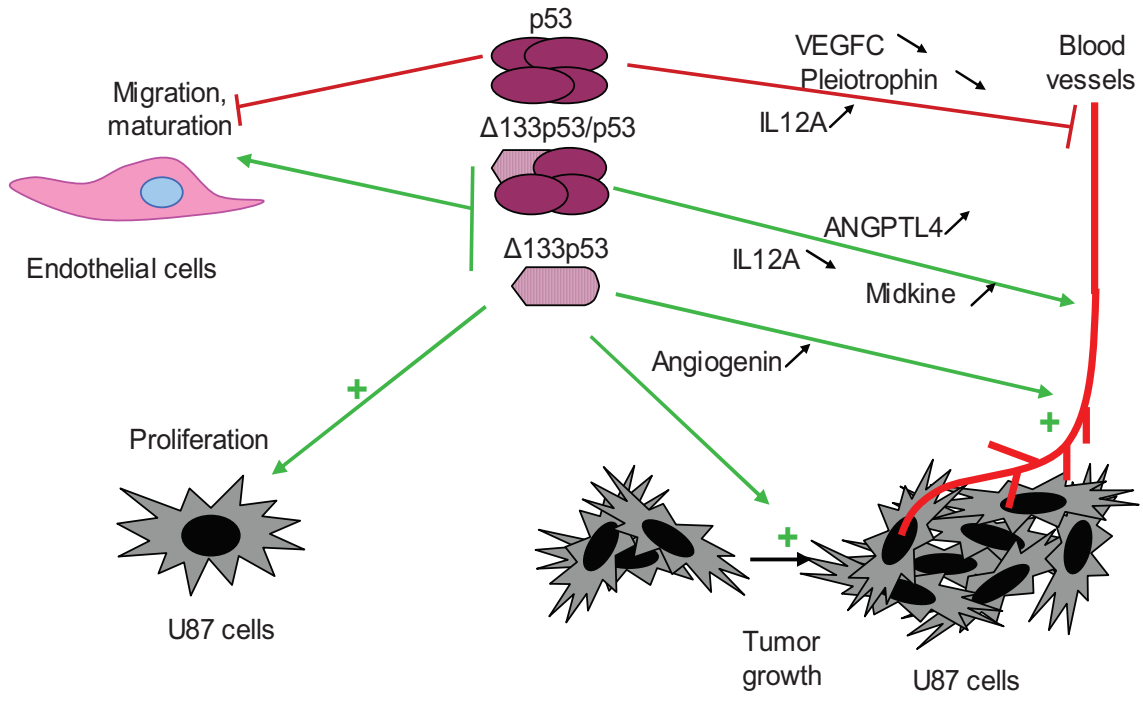
Figure 52. FGF-2 ELISA assay of conditioned media from U20S cells treated by SiRNAs. Experimental protocol for harvesting the conditioned media has been described in Figure 30.

CONCLUSION, DISCUSSION & PERSPECTIVES

The discovery of nine p53 isoforms constitutes a major breakthrough in p53 genetics and molecular biology, linking back its phylogenetic tree from zebrafish to human and unveiling several new modulators of p53 activity (Bourdon *et al.* 2005). Δ133p53 isoforms triplet i.e. α, β and γ do not possess the p53 transactivation domain but most of the DNA binding domain and, the ability to bind p53 and impair its antitumour potency (Fujita *et al.* 2009; Aoubala *et al.* 2010). Besides the dominant negative activity on p53, Δ133p53α isoform has shown selectivity in inhibition of p53 mediated transactivation. It is now established that following DNA damage p53 engages the Δ133p53α/p53 negative feedbackloop resulting in upregulation of Δ133p53α and inversely inactivation of p53.

In this manuscript I have focused my work to study p53 and Δ133p53 isoforms involvement in angiogenesis. I have demonstrated the major impact of Δ133p53 isoforms for the formation of new blood vessels, the settlement of ectopic tumours xenografts and its progression *in vitro* and *in vivo*. By Taqman low density array I have identified new target genes of p53 and Δ133p53 such as IL-12α, MMP2, Pleiotrophin, VEGF-C, HGF and ANGPTL4 and showed that Δ133p53 is capable to transactivate genes independently of p53 – Midkine and Angiogenin. These results are represented diagrammatically in Figure 55. Finally, I have shown that Δ133p53 disruption cause transactivation of p53 expression.

Figure 55. Schematic representation of Δ133p53 effects on angiogenesis



I also have investigated the regulation of FGF-2 by p53. Interestingly p53 activity over FGF-2 protein expression is much more complex than previously thought. It appears that FGF-2 is not regulated through transcription or bioavaibility in the extracellular domain, but more by a translational mechanism implying p53 activation triggered by stresses such DNA damage treatment. p53 activation may result from a post-translational modification.

A. Mechanistic considerations about the role of Δ133p53

A.1. Which step and what mechanism are blocked?

Knockdown of Δ133p53 *in vitro* clearly result in a specific phenotype of cancer cells resulting in the induction of endothelial cell migration and tubulogenesis, but not proliferation – Figure 24, 30, 31 and 32. In consequence the molecules secreted by the tumour in the environment must act upon the remodelling of the cytosqueleton, through a motility process including sequentially: formation of filopodia sensing angiogenic stimulus, extension of lamellipodia, focal adhesion attachment to extracellular matrix by focal adhesions, contraction of the cell body, rear release and recycling – Reviewed in (Lamalice *et al.* 2007). Quite surprisingly, TLDA results show that neither FGF-1 nor FGF-2 are downregulated when Δ133p53 or p53 levels are disrupted – Figure 27. In parallel, I have observed no variation of FGF-2 secretion in U87 cells – Figure 38. On the contrary, Angiogenin, which upregulates endothelial cell migration by interacting with α-actin and inducing plasmin activity, has its mRNA expression strongly decreased by Δ133p53 knockdown. α-actin, expressed in smooth muscle cells but also in U87 cell line, may be cleaved from the extra-cellular domain of U87 cells, proceed to conditioned media and activate the plasminogen cleavage in endothelial cell cultures (Kim *et al.* 2007). It is also able to induce proliferation through its 170 kDa receptor or induction of rRNA transcription. For these reasons, Angiogenin, identified the present study, seems to be a good candidate to explain how Δ133p53 isoforms can inhibit cell migration and proliferation, and consequently angiogenesis.

In the same assay, Midkine and heparin growth factor – HGF – mRNA levels are downregulated in SiΔ133p53 condition. Although these latter regulations have less

amplitude than the Angiogenin mRNA regulation, they can be determinant for the induction of anti-angiogenic phenotype. MMP-2 mRNA levels in both Δ133p53 and p53 knockdowns are upregulated suggesting a complex mechanism.

Finally, angiopoietin-like 4 protein is also strongly regulated by SiΔ133p53 and could be essential in antiangiogenic and antitumoural activity of Δ133p53. The results obtained in this work would strengthen the hypothesis of an angiogenic factor which complex regulation can have opposite consequences.

It is now important to confirm these results by expressing each Δ133p53 isoform in U87 and to analyse Δ133p53 pro-angiogenic and pro-tumoural effects in other cell lines and link these effects with specific secreted factors. For this reason we have developed lentivirus vectors expressing each Δ133p53 isoform in an inducible manner in order to tightly control their levels of expression and thus the ratio p53/Δ133p53 which appears for us very important in Δ133p53's activities.

A.2. Intrinsic versus extrinsic activity of Δ133p53

Taqman angiogenesis assay showed new target genes of p53 such as VEGF-C and pleiotrophin and concomitant new target genes of p53 and Δ133p53 such as IL-12α. It reveals a novel and interesting aspect of p53 in lymphangiogenesis, although only correlations have been established between p53 status and tumour dissemination through lymph nodes and lymph node metastasis (Ruddell *et al.* 2008).

More importantly, TLDA teaches us that Δ133p53 is able to transactivate angiogenin independently of p53. This aspect is confirmed by *in vivo* measurements of tumour growth in Δ133p53 knockdown and double Δ133p53/p53 knockdowns. These results show us that at least angiogenin but maybe other factors triggered by Δ133p53 are induced and facilitate tumour growth. Transcriptional activation domain is essential for p53 to have its basal transactivating activity, but Δ133p53 kept almost all the DNA binding domain sequence suggesting that Δ133p53 can still bind to DNA. Further investigation would permit us to see if Δ133p53 might have a transactivating activity by itself or or mediated by another partner.

A.3. p53 mRNA regulation by Δ133p53

I have shown that Δ133p53 knockdown induce p53 mRNA expression. This additional layer of regulation in the loop constituted by p53 and Δ133p53 link even more tightly these two partners together. Consequently, low levels of Δ133p53 act on p53 mRNA transcription but when Δ133p53 is overexpressed – particularly for instance induced by p53 – it inhibits p53 by direct binding in tetramers. This indicates that Δ133p53 acts by at leats two different mechanisms leading to p53 downregulation or inactivation, respectively. This regulation can be useful in response to an important stress inducing p53 activation, particularly to set back p53 to levels of unstressed cells. However in conditions of constitutive overexpression of Δ133p53 such as in cancers, p53's activity is deeply impaired. This results in some cancers in a wildtype p53 blocked by overexpression of Δ133p53. For this reason it is essential to take into account not only p53 but also Δ133p53 for the measurement of p53 potency. This approach could tell us about the real opportunity of using p53 mediated therapeutics.

As mentioned above it is possible that Δ133p53 has intrinsic activity resulting from a transactivation activity - possibly mediated by its interaction with other partners. Several factors control p53 mRNA expression such as E2F1, NF-κB and YY1 and NB1 (Furlong *et al.* 1996; Benoit *et al.* 2000; Choi *et al.* 2002). It could be interesting to investigate if expression of these factors is regulated by Δ133p53. Finally another mechanism of p53 mRNA expression regulation could happen through modulation of p53 mRNA stability by enhanced degradation of p53 mRNA. In such a case we can hypothesize that Δ133p53 would directly bind to the mRNA through its DNA binding domain and influence mRNA stability.

According to my results from TLDA, Δ133p53 intrinsic activity woud also exert itself on expression of specific angiogenic factors either by transactivation or by mRNA destabilization.

B. Mechanism of translation regulation by p53.

In this present study we have shown that endogenous p53 is able to modify the activity of FGF-2 IRES and its protein expression level, but not FGF-2 transcription – contrarily to previous work by Ueba *et al.* – nor its secretion – Figure 42, 44, 45 and 52. Hovever in normal culture conditions we could not show any regulation of FGF-2 protein expression by Δ133p53 – Figure 46. Such an apparent contradiction may be due

to the fact that FGF2 mRNA translation occurs by both cap- and IRES-dependent mechanisms. In unstressed conditions the cap-dependent translation of FGF2 mRNA would be predominant and mask any effect of p53 on the IRES-dependent translation of FGF2 mRNA. However, when cells are stressed by doxorubicin, we observe a transient inhibition of FGF2 protein expression related to p53 induction. Although we have not definitively proven that FGF2 protein downregulation is directly due to p53, these data suggest that translational regulation of FGF2 mRNA translation would occur in stress conditions, when the cap-dependent translation is blocked. This would provide a real physiological significance to the effect of p53 on IRES-dependent translation.

In previous reports we had already proposed the hypothesis that p53 could be a inhibitory ITAF able to inhibit translation by direct binding to the FGF2 mRNA (Galy *et al.* 2001). Here we have confirmed, by our knockdown approach, that endogenous p53 is indeed able block IRES-dependent translation, and that this effect is not limited to the FGF2 IRES as c-myc IRES is also regulated by p53. In this respect, molecular approaches such as BIAcore technology allowing us to identify and determine the quality of specific protein-mRNA interactions will be of interest to set the affinity of such interactions and elucidate the crosstalk between p53 and the other ITAFs such hnRNPAI defined as activators of the FGF2 IRES (Bonnal *et al.,* 2005).

ANNEX

In order to prove that human p53 upregulates Δ133p53 in response to stress, I did Doxorubicin treatments in U87 cells and observed the protein expression of the p53 isoforms – Figure 54. As we can see, even if doxorubicin induce increased levels of p53 protein, I could not reveal the Δ133p53 isoforms and long exposure for revelation did not show more information – Data not shown.

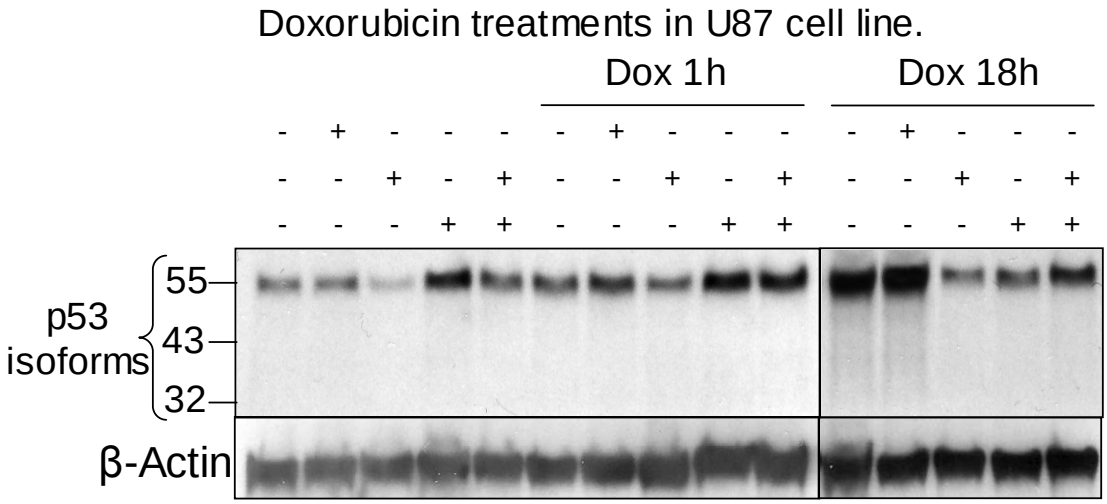


Figure 54. Western blot of p53 in U87 cells after treatment of 1µM of Doxorubicin. Either cells were subjected to 1 hour of dox and 17h of recovery, or continuous treatment of doxorubicin during 18 hours. p53 immunostaining with Sapu antibody and β-Actin with β-Actin antibody.

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